

*Annual Report of*  
*Intramural Research Program Activities*

*National Institute on Alcohol Abuse  
and Alcoholism*

*Fiscal Year 1994*

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

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*Annual Report of*  
*Intramural Research Program*  
*Activities*

*National Institute on Alcohol Abuse*  
*and Alcoholism*

*October 1, 1993 to September 30, 1994*

*Summary Statements and*

*Individual Project Reports*

**U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES**

**Public Health Service**

**National Institutes of Health**

**National Institute on Alcohol Abuse and Alcoholism**

**9000 Rockville Pike**

**Bethesda, MD 20892**





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Annual Report of the  
Division of Intramural Clinical and Biological Research  
. National Institute on Alcohol Abuse and Alcoholism  
October 1, 1993 to September 30, 1994  
Markku Linnoila, M.D., Ph.D., Scientific Director

In FY 1994, the NIAAA Intramural Research Program (IRP) has regained its footing despite the continuing hiring freeze, which has cut full-time employee positions from 117 to 102, and the continuing delay of planned and absolutely necessary renovations of laboratory space. Investigators in the IRP have been expanding the envelope of brain imaging studies by developing new ligands to investigate the serotonin system in the brains of human and nonhuman primates. Simultaneously, molecular genetic studies have identified polymorphisms which lead to coding sequence amino acid substitutions in gene products controlling serotonergic functions. In the near future, we will be able to combine these two powerful approaches. Then we will identify patients with known polymorphisms and do positron emission tomography (PET) imaging on them using ligands specific for the polymorphic proteins.

Collaboration with other IRPs on the NIH campus has been very active in the areas of human molecular genetics, brain imaging, nutrition, hepatitis C, and cognitive neuroscience research.

The major research themes of the IRP in FY 1994 have included brain imaging using PET to quantify neuronal activation within anatomically well-defined brain areas as determined by magnetic resonance imaging, molecular genetic mapping of candidate vulnerability genes within well defined alcoholic families, investigations on effects of alcohol on ion channel, and receptor functions and gene expression.

Studies on effects of ethanol on cell membrane receptors, ion channels, and expression of genes coding for these important proteins are yielding intriguing insights into basic mechanisms of ethanol's action. Combined with studies on region specific effects of ethanol on the release of neurotransmitters, these investigations will elucidate how ethanol produces reward, dependence, tolerance, and brain damage. Behavioral studies on specifically bred or genetically altered whole animals, using primarily mice and monkeys, combined with molecular genetics and behavioral manipulations during development, examine important protective and causal factors for alcohol abuse and dependence. These investigations have already pinpointed developmental factors in rhesus macaques which are conducive to alcohol abuse in this species.

The Intramural Research Program is a creative, vibrant, internationally known and highly productive research and training center which conducts studies at the cutting edge of science in several fast developing fields.





**LABORATORY OF CLINICAL STUDIES**



Annual Report of the  
Laboratory of Clinical Studies  
Division of Intramural Clinical and Biological Research  
National Institute on Alcohol Abuse and Alcoholism  
October 1, 1993 to September 30, 1994  
Markku Linnoila, M.D., Ph.D., Acting Chief

## Introduction

During Fiscal Year 1994, investigators in the Laboratory of Clinical Studies (LCS) continued the projects outlined in previous annual reports and initiated new studies.

The past year continued to be one of administrative transition. The Laboratory has maintained research productivity, initiated new studies, and has commenced long-range planning.

## Section of Cognitive Neurosciences

Subgroups of alcoholics express selective impairments in a wide array of cognitive functions that require reflection (impairments in monitoring and evaluative functions as well as operations that are under voluntary control that are used to control and allocate cognitive resources). In contrast, normal volunteers, including the elderly and many other types of neuropsychiatric disorder patients, are unimpaired in their ability to use reflective functions. Drug challenges are useful in delineating the types of cognitive impairment that are present in these different types of patients (i.e., a benzodiazepine challenge in detoxified alcoholics, stimulant and cholinergic drugs challenges in elderly normal volunteers and in Alzheimer's disease patients, ketamine in schizophrenic patients and normal controls, and DHEA administration in depression).

Acute administration of benzodiazepines stimulates the type of cognitive impairment (dysfunction in reflective (control) cognitive operations) in normal volunteers and potentiates the existing deficit in alcoholics. More specifically, normal volunteers, treated with the benzodiazepine triazolam, are unable to effectively, strategically, shift their attention, are not able to appreciate the extent to which they are sedated, can not monitor the accuracy nor the source of what they remember, and are unable to suppress errors in performing cognitive tasks despite the fact that many other facets of their cognitive functioning are spared. The effect of acute alcohol administration on cognition is similar to those expressed by the benzodiazepines, and different from other classes of drugs, i.e., cholinergic antagonists, but similar to the effects of the anesthetic ketamine, an antagonist of the NMDA-type glutamate receptor (a receptor believed to be involved in long-term potentiation (memory consolidation)). Drugs such as benzodiazepines, and alcohol in normal controls, also produce qualitative shifts in how both normal controls and alcoholics retrieve previously acquired knowledge. That is, these type of drugs alter retrieval context which is manifest in the specific facets of previously acquired experiences that can be recalled. This is apparent in the form of state (contest)-dependent retrieval of self generated information, source of knowledge, and evaluation of intrusion errors. The presence of these drugs at the time of retrieval can also enhance the amount of information that can be remembered for information acquired just prior to drug administration. The profile of highly specific effects of benzodiazepines (and alcohol) on reflective functions may be important in understanding patterns of uncontrolled drinking in alcoholics and also provide data on the stimulus discriminative (and reinforcing) properties of these drugs. This research is being extended to the study of alcohol craving and the study of children who are at risk for developing alcohol-related problems.

## Section of Brain Electrophysiology and Imaging

Investigators in the Section of Brain Electrophysiology and Imaging conduct sophisticated electrophysiological, neuropsychological, and brain imaging studies on alcoholics, individuals at risk, and carefully matched controls.

The lack of an acceptable method for determining statistical significance of differences in brain images derived from functional Magnetic Resonance Imaging (MRI) or Positron Emission Tomography (PET) studies has been a major problem for researchers in these areas. Over the past year we have made significant progress in applying rigorous statistical methods based on a Gaussian random field model to the analysis of image data. Using these techniques we have been able to demonstrate significant differences in glucose metabolism in the brains of young, cognitively unimpaired alcoholics and normal controls. These differences are localized to the right middle frontal gyrus and the mesial aspects of the superior frontal gyrus. The Gaussian random field technique also allows us to examine the correlations between regional brain metabolism and variables such as age, severity of alcoholism, or cognitive ability. Using this approach we found that while regional brain glucose metabolism does not decrease with age among normals, it does decrease among alcoholics. Brain glucose metabolism is significantly, negatively correlated with the age of alcoholics. The brain regions showing the highest correlations are the orbital and mesial frontal areas. This distribution of cortical regions affected in alcoholism is similar to the distribution of regions that when damaged in previously normal individuals leads to a syndrome of acquired sociopathy. Thus, the sociopathy characteristic of many older alcoholics may be secondary to a selective neurotoxicity of alcohol.

During the past year we have begun to use functional MRI scanning to investigate the effects of olfactory stimulation, including the smell of alcoholic beverages, on regional brain blood volume. We have found that pleasant odors produce a significant increase in blood volume in several limbic, secondary olfactory areas, including: the nucleus accumbens, amygdala, and hypothalamus. The goal of this research is to use olfactory stimuli to study the functional neuroanatomy of brain states associated with the desire for food or beverage alcohol.

Work on the development of advanced image analysis and coregistration for PET, CT, structural and functional MRI has continued. Methods to achieve the 3-D registration of PET images with structural MRI have been developed as have techniques for the automated detection of midsagittal lines or planes. Segmentation techniques are being extended for the automated labeling of CSF, white and gray matter regions in structural MRI data. Work on alternative methods for determining the statistical significance of differences observed in arbitrary regions of group average images are under further development. These include methods of spatial frequency decomposition as well as wavelet analysis. We are in the process of comparing the relative merits and shortcomings of these methods with Gaussian random field based techniques.

Over the past year the Section has focused on two areas of brain electrophysiology. The first has been an attempt to define electroencephalographic phenotypes for the use in genetic studies of alcoholism and related disorders. These studies have been conducted in collaboration with Dr. David Goldman of the Laboratory of Neurogenetics. In the past we have demonstrated that low voltage alpha EEG activity is transmitted as an autosomal dominant in certain families. We have continued to study families to confirm our previous observations and to determine if phenotypes based on evoked potential latencies or amplitudes can also be identified. In addition we are beginning to apply Laplacian transforms to scalp potentials in order to determine surface current densities. Current density measurements provide much greater spatial resolution than can be obtained with traditionally evoked potential measurements.

## Section of Clinical Science

The major objectives of research conducted in the Section of Clinical Science are to: (1) characterize the role of various neurotransmitter systems in the etiology of alcoholism by utilizing cerebrospinal fluid metabolite determinations, pharmacological challenge paradigms, and PET studies; (2) explore possible biochemical determinants that might differentiate subtypes of alcoholic patients; (3) describe and understand the behavioral and biochemical interactions among alcoholism, panic disorder, and depression; (4) explore gender differences in alcoholism by using a serotonin challenge paradigm; (5) investigate the role of diet, and carbohydrate consumption in particular, as possible determinants of alcohol consumption; (6) use procaine as a probe for limbic system function in alcoholics with and without panic disorder; (7) introduce new pharmacological interventions for long-term treatment of alcoholism; (8) study the psychological and biological effects of smoking cessation in detoxifying alcoholics; and (9) characterize the concept of "losing control" as it relates to violent behavior and alcoholic drinking.

Results from pharmacological-challenge paradigms have been particularly interesting. Administration of the serotonergic partial agonist m-chlorophenylpiperazine (m-CPP) to detoxified alcoholics resulted in different responses from early-onset (Type II) and late-onset alcoholics (Type I), with early-onset alcoholics more likely to report a "craving" for alcohol, while the late-onset alcoholics reported more anxiety. Possible differences in serotonergic functions between subtypes and alcoholics were substantiated by cerebrospinal fluid analyses. Abstinent alcoholics who had the onset of alcoholism before the age of 25 had lower 5-HIAA concentrations compared to patients who had the onset of alcoholism after the age of 25. Administration of either 5-HTP or L-DOPA compared to placebo had no effect on the length of time between detoxification and relapse to alcohol consumption. Results from other challenge paradigms suggest that there may be other biological differences between alcoholics and controls. Following the administration of the physiological stressor 2-deoxyglucose (2-DG), alcoholics showed an exaggerated ACTH response compared to controls. Both baseline insulin as well as insulin release (AUC) following 2-DG showed a significant positive correlation with the quantity of alcohol consumed per occasion during the last six months. Administration of sodium lactate to detoxified alcoholics with panic disorder resulted in a lower frequency of panic attacks compared to panic patients without alcoholism. This difference was not explained according to whether the panic disorder started before or after the onset of alcoholism. Administration of dextrose in lactate decreased the likelihood of panic subjects experiencing a lactate-induced panic attack. Intravenous administration of the local anesthetic procaine resulted in an increased frequency of panic attacks in panic patients (with and without alcoholism) compared to alcoholics and controls. Those patients having a panic reaction had a greater rise in pulse and epinephrine release compared to those not having a panic attack.

Preliminary results indicate that most alcoholics are able to be successfully withdrawn from nicotine while being treated for alcoholism in a controlled environment. Physiological results from an idoxan challenge shows little evidence to suggest altered  $\alpha_2$  receptor activity during smoking withdrawal.

Characterization of individuals who "lose control" and become physically violent have an increased prevalence of panic disorder/attacks and alcoholism/abuse compared to the general population. Approximately one-half of those studied experienced a sense of "losing control" and/or fear of becoming violent during a lactate challenge study. Three out of four patients who had a PET scan demonstrated a decrease in glucose metabolism in the orbital-frontal brain regions.



### Section on Neurochemistry and Neuroendocrinology

The Section on Neurochemistry and Neuroendocrinology has continued research on biochemical concomitants of violent behavior in alcoholics and on variables associated with increased vulnerability of developing alcoholism-related behavior. The major focus of this research has remained the serotonergic system. Interesting insights have been gained into regulation of serotonergic neuronal networks, developmental and genetic influences on serotonin functions, and serotonergic regulation of energy metabolism and excessive alcohol consumption.

### Section of Clinical Assessment and Biological Correlates

The Section of Clinical Assessment and Biological Correlates clinically evaluates subjects for genetic, physiological, and biochemical studies conducted by other investigators in the Laboratory and the Division, as well as studies within the Section itself. Research currently in progress includes studies comparing different subgroups of alcoholics, both men and women, in order to elucidate risk factors for alcoholism and impulsive behaviors such as suicide attempts, physical violence, and drug abuse. There is an additional focus on clinical personality traits within these subgroups. The Section is also in the process of assessing psychiatric diagnoses and characteristics of alcoholism in American Indians, Blacks, and Caucasians (Americans and Finns); collaborations with other groups are predominant.

Developmental studies involving aggressive, impulsive, and conduct-disordered children are underway, both within the Laboratory and in collaboration with the Laboratory of Developmental Psychology, NIMH.

This Section has initiated the use of computer-assisted programs in diagnostic assessments of both adults and children.

### Unit of Pharmacokinetic Studies

Research efforts of the Unit of Pharmacokinetic Studies primarily focused on the development of appropriate kinetic models to describe and quantify the movement of various tracers to be utilized in PET studies. alpha-methyl-L-tryptophan continues to be developed as a serotonin PET ligand that will be used to determine differences in the activity of the serotonergic system in aggressive and non-aggressive non-human primates and humans.

Research concerning the determination of the kinetics of new therapeutic agents that are being evaluated as treatments in non-human primates for anxiety-mediated and/or stress and non-stress conditions is also being pursued.

Another area of concern is determining how the acute and chronic effects of alcohol alter P450 mechanisms. Initial research has focused on cytochrome P450 isozymes and membrane structure in rats during chronic ethanol exposure and during acute and protracted withdrawal. Animal studies will guide the development of human research protocols.

Publications  
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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 AA 00058-03 LCS																																
PERIOD COVERED <b>October 1, 1993 to September 30, 1994</b>																																		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Protracted Withdrawal from Alcohol</b>																																		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">M. Eckardt</td> <td style="width: 35%;">Senior Investigator</td> <td style="width: 15%;">LCS, NIAAA</td> </tr> <tr> <td>Others:</td> <td>P. Andreason</td> <td>Senior Clinical Investigator</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>G. Brown</td> <td>Clinical Director</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>D. George</td> <td>Senior Clinical Investigator</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>S. Goodson</td> <td>Senior Staff Fellow</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>D. Herion</td> <td>Senior Staff Fellow</td> <td>LCS, NIAAA</td> </tr> <tr> <td></td> <td>M. Linnoila</td> <td>Scientific Director</td> <td>NIAAA</td> </tr> <tr> <td></td> <td>W. Williams</td> <td>Senior Clinical Investigator</td> <td>LCS, NIAAA</td> </tr> </table>			PI:	M. Eckardt	Senior Investigator	LCS, NIAAA	Others:	P. Andreason	Senior Clinical Investigator	LCS, NIAAA		G. Brown	Clinical Director	LCS, NIAAA		D. George	Senior Clinical Investigator	LCS, NIAAA		S. Goodson	Senior Staff Fellow	LCS, NIAAA		D. Herion	Senior Staff Fellow	LCS, NIAAA		M. Linnoila	Scientific Director	NIAAA		W. Williams	Senior Clinical Investigator	LCS, NIAAA
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	M. Linnoila	Scientific Director	NIAAA																															
	W. Williams	Senior Clinical Investigator	LCS, NIAAA																															
COOPERATING UNITS (if any) Clinical Pathology, CC, NIH (R. Elin); BPB, NIMH (P. Gold, D. Michelson); Diagnostic Radiology, CC, NIH (J. Doppman)																																		
LAB/BRANCH <b>Laboratory of Clinical Studies</b>																																		
SECTION <b>Office of the Chief</b>																																		
INSTITUTE AND LOCATION <b>NIAAA, 9000 Rockville Pike, Bethesda, MD 20892</b>																																		
TOTAL STAFF YEARS: <div style="text-align: center;">1.0</div>	PROFESSIONAL: <div style="text-align: center;">0.5</div>	OTHER: <div style="text-align: center;">0.5</div>																																
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																																		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Male and female alcoholics are being evaluated repeatedly during the course of withdrawal from alcohol for changes in brain neurotransmitters and neuropeptides, hypothalamic-pituitary-adrenal axis (HPA) functioning, immunocompetency, brain structure, cognition, magnesium, zinc, and activity levels.																																		

Project Description:Investigators:

M. Eckardt	Senior Investigator	LCS, NIAAA
P. Andreason	Senior Clinical Investigator	LCS, NIAAA
G. Brown	Clinical Director	LCS, NIAAA
J. Doppman	Chief	DR, CC
R. Elin	Chief	CP, CC
D. George	Senior Clinical Investigator	LCS, NIAAA
P. Gold	Chief	BPB, NIMH
S. Goodson	Senior Staff Fellow	LCS, NIAAA
D. Herion	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
D. Michelson	Senior Staff Fellow	BPB, NIMH
W. Williams	Senior Clinical Investigator	LCS, NIAAA

Objectives:

The intent of this project is to characterize the pathophysiologic concomitants of withdrawal from alcohol in individuals that do not require medication. Males and females will be studied repeatedly over an eight-week period in order to determine the time course of recovery. Specific attention will be focused on brain neurotransmitters and neuropeptides, hypothalamic-pituitary-adrenal axis functioning, immunocompetency, brain structure, cognition, magnesium, zinc, and activity levels.

Methods Employed:

Male and female patients admitted to the study meet DSM III-R criteria for alcohol dependence and have consumed alcohol within the past 48 hours. Patients with severe cardiovascular, kidney, liver, gastrointestinal or endocrine disease are excluded from the study. Patients with organic brain syndromes or a history of substance abuse/dependence or major psychiatric disorder are also excluded.

Patients follow a low monoamine diet throughout the study and mood and craving analogue scales are administered daily throughout the eight-week study. CSF samples are obtained on admission and one and six weeks later; levels of neurotransmitters and neuropeptides are determined. Magnesium levels are determined on admission and one and four weeks later. Activity levels are measured on admission and one and six weeks later. Volumes of brain and adrenal gland are determined by MRI on admission and after six weeks. Cortisol levels (plasma and urine) are determined within 48-72 hours after admission and one and six weeks later. Electrical activity of the brain (EEG and event-related potentials) and cognition are evaluated on admission and four weeks later. Lastly, ACTH and oCRH challenges are conducted during the seventh week.

Major Findings:

Data are still being collected.

Significance to Biomedical Research and the Program of the Institute:

Alcohol withdrawal and recovery to normal functioning have not been well characterized quantitatively. It is important to establish the time parameters required to return to "normal" in order to develop new cost-effective treatments to accelerate recovery. In that only patients who do not require medication to treat withdrawal signs and symptoms will be studied, the present project also provides a description of social detoxification. Because there are few facilities that are able to study patients for extended periods of time, this study offers unique promise.

Proposed Course:

Data will continue to be collected.

Publications:

None.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 AA 00231-12 LCS
PERIOD COVERED <b>October 1, 1993 to September 30, 1994</b>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Central and Peripheral Nervous System Function in Abstinent Alcoholics</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <b>PI: M. Eckardt Senior Investigator LCS, NIAAA</b>  <b>Others: D. George Senior Clinical Investigator LCS, NIAAA</b> <b>M. Linnoila Scientific Director NIAAA</b>		
COOPERATING UNITS (if any) Vanderbilt University (P. Martin); VAMC, Charleston, SC (B. Adinoff); Clinical Neuroendocrinology Branch, NIMH (P. Gold)		
LAB/BRANCH Laboratory of Clinical Studies		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NIAAA, 9000 Rockville Pike, Bethesda, MD 20892		
TOTAL STAFF YEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input checked="" type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Behavioral deficits in alcoholics have been conceptualized in terms of two neuropathologically distinct syndromes: alcoholic dementia and Korsakoff's psychosis (alcohol amnesic disorder). Alcoholic dementia is characterized by diffuse cortical damage primarily related to the neurotoxicity of alcohol; Korsakoff's psychosis is associated with subcortical lesions due to nutritional (thiamine) deficiency. Severe memory impairment with relative sparing of other intellectual functions distinguishes Korsakoff's psychosis from alcoholic dementia (which may be clinically indistinguishable from the most common cause of dementia, Alzheimer's disease). We have reported that sleep in Korsakoff patients is characterized by a reduced REM latency compared to normal volunteers, whereas Alzheimer patients have normal latencies. Furthermore, delta sleep is reduced in Alzheimer's disease, but is normal in Korsakoff's psychosis. Most patients with demonstrated reduced daily excretion of the major urinary metabolite of melatonin, hydroxymelatonin, present with Korsakoff's psychosis. This finding is suggestive of impaired pineal function. Genetic differences in thiamine metabolism may predispose patients to develop Korsakoff's psychosis. Most patients with Korsakoff's psychosis whom we have studied have had a transketolase with reduced affinity for thiamine pyrophosphate. Modifying activation and arousal by pharmacologic modulation of neurotransmitter systems may be effective in treatment of Korsakoff's psychosis, whereas alcoholic dementia may require treatment strategies similar to those in Alzheimer's disease. This protocol is intended to utilize clinical, neuroradiological, physiological, and neuropharmacological studies to differentiate these two pathologic entities, to follow a longitudinal course, and to relate variables in treatment protocols to outcome.</p>		

Project Description:Investigators:

M. Eckardt	Senior Investigator	LCS, NIAAA
D. George	Senior Clinical Investigator	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA

Objectives:

Chronic organic brain syndromes due to alcoholism constitute the second most common cause of dementia in adults (approximately 10%), ranking next to senile dementia of the Alzheimer's type (40-60%). Currently, a large proportion of dementing illness can be diagnosed with certainty only by examining the microscopic structure of the brain at autopsy. The cross-sectional clinical picture of alcohol-related cognitive decline may be difficult to distinguish from that of the more prevalent primary degenerative dementia, Alzheimer's disease. Chronic alcohol abuse may lead to two clinically and neuropathologically distinguishable syndromes: alcoholic dementia and alcohol amnestic syndrome (also called Korsakoff's psychosis). These two organic brain syndromes may represent extremes of the spectrum of cognitive impairments related to chronic alcoholism. Alcoholic dementia is characterized by global intellectual decline, whereas the salient clinical feature of the alcohol amnestic syndrome is a severe and persistent memory deficit with relative sparing of other intellectual functions. The majority of alcoholic patients have aspects of both syndromes; presumably the midline subcortical lesions due to thiamine deficiency may explain the amnestic component, where the diffuse bilateral cortical damage resulting from alcohol neurotoxicity explains the global cognitive loss. It has been postulated that polymorphisms of thiamine-requiring enzymes may influence which clinical syndrome predominates. Most of the patients with Korsakoff's psychosis in whom we have studied fibroblast transketolase have had an elevated  $K_m$  for thiamine pyrophosphate in comparison with the  $K_m$  derived from normal controls. We have found that patients with relatively "pure" amnestic characteristics have demonstrated episodic memory impairments that resemble those found in depression and Parkinson's disease and are distinguishable from the semantic or knowledge memory deficits found in Alzheimer's disease. Furthermore, we have demonstrated significant differences in the pattern of sleep EEG abnormalities in Korsakoff's psychosis patients compared with those with Alzheimer's disease. The sleep of Korsakoff patients resembles that of patients with depression (increased arousal and shortened REM latency). We postulate that treatment strategies directed toward modifying activation and arousal by pharmacologic modulation of neurotransmitter systems may be effective in treatment of the alcoholic amnestic syndrome. This situation is analogous to the benefits derived from pharmacotherapy on depression and Parkinson's disease, whereas alcoholic dementia requires treatment approaches similar to those in Alzheimer's disease.

Methods Employed:

We will study two groups of controls (healthy nonalcoholics and alcoholics abstinent for at least six months) and four groups of patients (detoxified alcoholics who have been abstinent from alcohol for at least one week; alcoholics withdrawn from alcohol who have been abstinent for at least three weeks; alcohol amnestic patients; and alcoholic dementia patients), using the following clinical, physiological, and neurochemical tests: (1) neuropsychological evaluation of patients to determine whether they are predominantly amnestic or demented (in collaboration with Dr. Weingartner); (2) norepinephrine response to orthostasis; (3) dose-response to norepinephrine infusion; (4) norepinephrine and



endocrine responses to insulin tolerance test; (5) catecholamine and neuropeptide metabolism in cerebrospinal fluid versus plasma and urine; (6) vasopressin response to hypertonic saline infusion; (7) thyrotropin-releasing hormone and gonadotropin-releasing hormone stimulation tests (in collaboration with Dr. Gold); (8) circadian rhythms of melatonin, body temperature, and activity; and (9) sleep EEG.

#### Major Findings:

A number of potentially important findings have emerged. These are as follows: (1) In the saline infusion (vasopressin test), it appears that middle-aged alcoholics have blunted responses of vasopressin to the saline infusion. In other words, the normal age-related increase in vasopressin response to saline is considerably less in alcoholics. This would suggest damage to central hypothalamic mechanism, possibly as a result of drinking, since the young alcoholics do not show a similar pattern. (2) The previously reported blunting of response to the low rate of norepinephrine infusion was confirmed. Since plasma norepinephrine concentrations did not differ between these two groups, we conclude there is a subsensitivity in the alcoholics. However, the importance of this is hard to determine since, with the higher rates of infusion, alcoholics behave similar to controls. Analysis of heart changes during the norepinephrine infusion suggests that central baroreceptor mechanism are not altered in young alcoholics. (3) The insulin challenge test has demonstrated that amongst the alcoholic population there are two groups. One shows enhanced responsiveness to insulin, i.e., increased medullary responses, while the other shows a blunting of this response. Analysis of the pituitary hormone response to insulin-induced hypoglycemia is still pending. (4) Analysis of the pituitary response to the TRH/LHRH infusion has suggested that a number of alcoholics may be borderline hypothyroid. (5) The CRH challenge showed clearly that in alcoholics given CRH one week after withdrawal, the majority had markedly blunted ACTH responses. This may reflect adrenal hyperplasia in alcoholics, possibly secondary to the effects of withdrawal. In those subjects in which testing was performed after a long period of sobriety (up to several years in one patient), a consistent increase in ACTH response to CRH was observed. This suggests that alcohol has a profound attenuating effect on hypothalamic responsiveness to CRH and that many years may be necessary before full return to normal function is accomplished.

#### Significance to Biomedical Research and the Program of the Institute:

Chronic organic brain syndromes due to alcoholism are responsible for approximately 10% of dementia in the adult population. The fact that only a small population of alcoholics develop complications of alcoholism suggest the importance of predisposing factors. We will attempt to identify genetic factors that may be predictive of which individuals will develop the alcohol amnesic syndrome if they abuse alcohol. We plan to develop a clinical, physiological, and biomedical classification system of alcoholism-associated chronic organic brain syndromes; this system will have diagnostic, prognostic, and therapeutic applications.

The findings of abnormal peripheral sympathetic responsiveness, especially that of the adrenal gland in young alcoholics, may help us understand the damage produced by alcohol. However, they may also reflect a trait predisposition in alcoholics which could conceivably be a biological marker for the risk of alcoholism and be related to the etiology of alcoholism. Clearly, we need to study nonalcoholic adult children of alcoholics to determine whether similar abnormalities exist, and thus to establish or refute whether this represents an inherited contribution to the disease. Further findings suggest that in most recently abstinent alcoholics, the total ACTH response to CRH stimulation is decreased, and that this response tends to "normalize" over time. Changes in the correlation between baseline cortisol levels and HPA axis responses to CRH were evident even in alcoholics with greater than six months' abstinence. This suggests that alternations in HPA axis functioning may persist for prolonged

periods following cessation of drinking. It is not clear from this study if these findings are due to chronic effects of ethanol or to effects of the subsequent withdrawal syndrome.

Although elevated baseline-free cortisol levels were not observed in any of the alcoholic groups studied, cortisol response to CRH stimulation was proportionately greater in the one week- and three week-abstinent low ACTH responders compared to controls. This observation is compatible with the development of hyperplasia and hyperresponsiveness of the adrenal cortex, which is known to occur after even a few days of stimulation with exogenous ACTH or with the application of experimentally-induced stress. As the group of alcoholics we tested at one week- and three week-abstinence had experienced no more than mild withdrawal symptoms, were in relatively good health, and were not tested until one week following their last drink, the findings reported are most likely quite subtle compared to the changes one might observe in a poorly nourished alcoholic following delirium tremens. Our observations may, therefore, suggest that pituitary responsiveness may be severely impaired for a prolonged period of time following abstinence in more severely impaired alcoholics and could contribute to the medical and psychological problems seen in this group.

Proposed Course:

A number of components of the projects have now been completed. A detailed analysis of the CSF variables has been accomplished. Relationships between selected CSF variables and drinking history and age of onset of alcoholism are currently being analyzed.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00239-11 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alcoholism-Associated Cognitive Impairment and Organic Brain Syndromes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Eckardt Senior Investigator LCS, NIAAA

Others: R. Rawlings Mathematical Statistician LCS, NIAAA  
H. Weingartner Section Chief LCS, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.50

PROFESSIONAL:

0.25

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this study is to examine the neuropsychological performance of several clinically defined populations of detoxified alcoholics. Comparisons will be made among detoxified alcoholics with clinically defined chronic organic brain syndromes, dementia or amnesic syndrome, less cognitively impaired alcoholics who are in alcoholism treatment programs, and nonalcoholic controls.

Project Description:Investigators:

M. Eckardt	Senior Investigator	LCS, NIAAA
R. Rawlings	Mathematical Statistician	LCS, NIAAA
H. Weingartner	Section Chief	LCS, NIAAA

Objectives:

Chronic alcohol abuse may lead to two clinically and neuropathologically distinguishable syndromes: alcoholic dementia and alcohol amnestic syndrome (called Korsakoff's psychosis), which together constitute the second most common cause of dementia in adults (approximately 10%). These two alcohol-related organic brain syndromes may represent the extremes on a cognitive dysfunction scale with alcoholic dementia characterized by a global intellectual decline, whereas alcohol amnestic syndrome can be characterized as a severe and persistent amnesia with a relative sparing of other intellectual functions. The majority of alcoholic patients in clinical practice fall somewhere in between. In the present study, we used a comprehensive battery of neuropsychological tests to differentiate alcoholic dementia from alcohol amnestic syndromes. Less cognitively impaired alcoholics and normal, age-matched volunteers were evaluated similarly. Comparisons among these groups will lead to a better characterization of cognitive similarities and differences in these groups.

Methods Employed:

Two clinically defined groups of alcoholics will be evaluated: participants in an alcoholism treatment program and those with sufficient, clinically defined cognitive impairment so as to be judged as not likely to benefit from treatment. The latter group will be separated by neuropsychological performance into those with alcoholic dementia and those with alcohol amnestic syndrome.

It has been shown previously that the neuropsychological performance of neurologically impaired alcoholics with dementia can be differentiated from that of alcoholics with amnestic syndrome. We plan to use a more comprehensive and sensitive test battery to better understand this differentiation.

The neuropsychological test battery is designed to obtain a global assessment of cognitive skills, an in-depth examination of memory functions, and an assessment of alcoholism-related cognitive decrements. The examination will take about 12 hours to complete. The battery consists of Halstead-Reitan Battery including Trails A and B; Wechsler Adult Intelligence Scale; Wechsler Memory Scale; Wisconsin Card Sorting Test; and selected memory tests designed to compare episodic versus semantic learning, automatic versus effortful learning, and language versus non-language learning.

Data were also collected on socioeconomic status, personality, childhood history of attention deficit disorder and hyperactivity, and drug use history, including alcohol.

Major Findings:

Data have been collected and analysis continues.

Significance to Biomedical Research and the Program of the Institute:

It has been well documented that alcoholics have impaired brain function. The present study is designed to better characterize the cognitive deficits observed in alcoholics so that more appropriate pharmacological intervention will be possible.

Proposed Course:

As analyses are completed, the results will be published in appropriate scientific journals.

Publications:

None.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00240-15 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cognitive Function in Male Alcoholics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Eckardt Senior Investigator LCS, NIAAA

Others: R. Rawlings Mathematical Statistician LCS, NIAAA

COOPERATING UNITS (if any)

TRISARD, Bethesda, Naval Hospital, Bethesda, MD (D. Groden); Neurology Service, Brooklyn VA Medical Center (J. Stapleton)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.50

PROFESSIONAL:

0.25

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This series of studies is concerned with cognitive function in detoxified male alcoholics. Recent and chronic alcohol consumption variables were found to interact with each other and with age and education in a non-linear fashion in predicting neuropsychological performance. Increased consumption predicted decreased performance, even on tests whose mean scores were in the normal range. Little or no improvement in performance was demonstrable with short-term abstinence (14-20 days), although long-term abstinence (seven months) was associated with improvement. Similarly, hepatic and hematologic characteristics of long-term abstainers improved, whereas these systems functioned abnormally in people who continued to consume alcoholic beverages, albeit at significantly reduced levels. Increased risk of relapse was associated with excessive drinkers who were relatively early in their alcoholic careers as assessed by years of abusive drinking and accumulated lifetime exposure to alcohol. Although statistically significant relationships were observed between scores on certain neuropsychological tests and post-treatment alcohol consumption, neuropsychological evaluation was determined to be of limited clinical utility.

Project Description:Investigators:

M. Eckardt	Senior Investigator	LCS, NIAAA
D. Groden	Chief	TRISARD, BNH
R. Rawlings	Mathematical Statistician	LCS, NIAAA
J. Stapleton	Investigator	Brooklyn UAMC

Objectives:

The present series of studies was designed to document the presence of cognitive impairment in male alcoholics, discern possible etiological factors related to this impairment, and determine whether improvement in function is associated with subsequent abstinence. Additional questions concern whether treatment should commence immediately after detoxification, relationships between cognitive function and treatment outcome, and neuropsychological consequences of post-treatment alcohol consumption.

Methods Employed:

A battery of neuropsychological tests was administered to drug-free alcoholic inpatients (n=91) within seven days of their last drink and again 17 days later. To control for practice effects, a nonalcoholic medical control group (n=20) also took the test battery twice, with approximately the same interval elapsing between administrations. Another group of alcoholic inpatients (n=32) took the tests only once, 14-31 days after their last drink. After patients completed the 21-day treatment program, they were contacted on a monthly basis to determine drinking behavior. At the end of seven months, they returned to the hospital. Before testing, a breathalyzer and/or clinical laboratory determination of blood alcohol level was carried out in an attempt to ensure sobriety during testing. The cognitive tests were then administered in a random order. Self-administered questionnaires were used to calculate post-treatment frequency of drinking alcohol and quantity consumed per occasion. Patient-supplied collaterals were then contacted to verify the patients' self-reports. Approximately 24 months after entrance into the treatment program, 17 of the original 91 patients were located and agreed to take the entire battery of neuropsychological tests again.

Another study was designed to investigate cognitive functioning in detoxified alcoholics who had alcohol-related problems for a relatively brief time. The first goal of this study was to determine multivariate relationships among recent and chronic alcohol consumption patterns, age, education, current emotional status, and neuropsychological performance. The second goal was to determine relationships among childhood symptoms of hyperactivity-minimal brain dysfunction, extent of familial alcoholism, and neuropsychological performance.

Major Findings:

Cognitive performance in drug-free alcoholic men is significantly predicted by chronic and recent drinking practices. Furthermore, it appears that certain patterns of consumption may accelerate the alcohol-induced decline of brain function. Little or no improvement in cognitive performance was demonstrable with short-term abstinence when controls were included for the effects of repeated testing. Continued alcohol consumption by recovering alcoholics is associated with reduced cognitive performance, while those who abstained have improved test scores. Neuropsychological performances determined 24 months after entrance into the program were at the same levels as at seven months after entrance. Similar findings were observed in clinical laboratory tests, with long-term abstainers (seven months) having improved hepatic and hematologic functioning in contrast to the continued abnormal functioning observed in those people who continued to drink, albeit at significantly reduced levels. Further analysis of these clinical laboratory tests revealed widespread and persistent



alcoholism-related alterations in organ system functioning, even after long-term abstinence (seven or 24 months).

Male alcoholics' pre-treatment levels of alcohol consumption were found to be related statistically to post-treatment levels of consumption with an increased risk of relapse associated with excessive drinkers who were relatively early in their alcoholic careers, as assessed by years of abusive drinking and accumulated lifetime exposure to alcohol.

Statistically significant relationships were observed between neuropsychological test scores and post-treatment alcohol consumption determined eight months after completing treatment for 72 alcoholics. These results, however, were influenced by the particular measure of post-treatment consumption evaluated, type of statistical analysis, and whether the entire sample of 72 or a subsample of 55 with consistently reported drinking information was used. Scores of tests most consistently showing relationships were often counter to the notion that increased neuropsychological performance predicts more favorable treatment outcome. Discriminant analysis resulted in 70% correct classification, with chance being 50%. It is concluded that neuropsychological evaluation is of limited clinical utility in predicting post-treatment alcohol consumption.

Only four of the 101 alcoholics evidenced mild cognitive dysfunction. Current psychiatric condition, state of anxiety and depression, and liver dysfunction were not related to cognition. Relationships among cognition and lifetime estimates of alcohol consumption (average of 189 kg) and number of days from last drink to testing were determined to be nonlinear and suggested that increased lifetime consumption was predictive of decreased performance and increased abstinence predicted better performance. Neither extent of familial alcoholism nor number of childhood signs and symptoms of hyperactivity-minimal brain dysfunction were predictive of cognition except that increased antisocial behavior predicted decreased cognition. It was concluded that cognition in young alcoholics averaging six years of excessive alcohol consumption was within normal limits, even though increased lifetime consumption predicted decreased test scores and increased abstinence predicted increased scores.

#### Significance to Biomedical Research and the Program of the Institute:

Recent and chronic drinking practices appear to have adverse effects on brain function in male alcoholics. Insofar as decisions about the initiation of therapeutic interventions which rely on cognitive processes are based on neuropsychological performance, we conclude that treatments may commence as soon as the clinical symptoms associated with acute withdrawal have subsided. Continued alcohol consumption by recovering alcoholics might serve to maintain cognitive performance at reduced levels, and this possibility should be considered in determining appropriate treatment goals for alcoholic patients. Neuropsychological evaluation is of limited clinical utility in predicting post-treatment alcohol consumption. However, it may be of value in assisting treatment staff in obtaining cognitively appropriate post-treatment employment for patients and in more effectively individualizing treatment, but this remains to be demonstrated.

#### Proposed Course:

Project is completed.

#### Publications:

Eckardt MJ, Stapleton JM, Rawlings RR, Davis EZ, Grodin DM. Neuropsychological functioning in detoxified alcoholics between 10 to 35 years of age, *Am J Psychiatry*, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00279-05 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Psychopathology in African American Alcoholics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. Moore Social Worker LCS, NIAAA

Others: G. Brown Clinical Director LCS, NIAAA  
I. Culver Psychologist LCS, NIAAA  
M. Eckardt Senior Investigator LCS, NIAAA  
M. Linnoila Scientific Director NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study will focus on psychopathology in African American alcoholics as compared to Caucasian alcoholics matched by age, gender, and social class. The information will be used to examine three areas: prevalence of anxiety disorders among alcoholics; is there a difference in the type of anxiety disorder manifested by males vs. females?; and, does the date of onset of anxiety disorder vs. alcoholism alter the incidence and severity of alcoholism?

The children and adolescent study will focus on the identification of similar psychopathology, however, with a focus on resilience as well as protective factors in children of nonalcoholics with and without other psychopathology. Possible resilient factors that will be studied include family relationships and home environment (Family Assessment Device), locus of control, self-esteem, and academic performance. The study will also include children diagnosed with ADHD. Parents will be evaluated for alcoholism vs. Attention Deficit Disorder-Residual Type.

This project was previously titled "Black and white offspring of alcoholic vs. nonalcoholic parents".

Project Description:Investigators:

V. Moore	Social Worker	LCS, NIAAA
G. Brown	Clinical Director	LCS, NIAAA
I. Culver	Psychologist	LCS, NIAAA
M. Eckardt	Senior Investigator	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA

Objectives:

Investigations in the area of research and theory on psychopathology in African Americans (Bulham, 1985) are chaotic and contradictory. Approaches indicate foci, different methods, and diverse perspectives. Very few of these studies consist of controlled matched samples. Most research on the etiology of alcoholism was completed on populations not containing a representative sample of African American participants (Frances and Franklin, 1988). Consequently, African Americans have been misdiagnosed as having serious psychiatric problems that were in fact neuropsychiatric complications of alcoholism, such as organic brain syndrome (Bell, Thompson et al., 1985). Rimmer, Pitts, Reich, and Winolur's (1971) findings reveal that African American abusers had more medical complications than Caucasians, a higher frequency of psychiatric comorbidity, and a higher prevalence of additional drug use. Recent focus in the research literature has been on the comorbidity of specific disorders and alcoholism. The present study compares matched samples of African American and Caucasian alcoholics matched by age, gender, and social class to determine psychopathology, comorbidity, and the effects of age of onset on alcoholism.

Methods Employed:

The sample was defined on the basis of satisfying Research Diagnostic Criteria (RDC, Spitzer et al, 1975) for alcoholism and was derived from all African American individuals who agreed to participate in research conducted by the National Institute on Alcohol Abuse and Alcoholism on the campus of the National Institutes of Health in Bethesda, Maryland. All alcoholics were administered the Schedule of Affective Disorders and Schizophrenia-Lifetime Version (SADS-L; Spitzer and Endicott, 1979) by a social worker and subsequently blind-rated by another social worker and a psychiatrist; all diagnoses were reviewed and any inconsistencies were resolved by a senior psychiatrist. The Hollingshead-Redlich four Factor Index of Social Position, Michigan Alcoholism Screening Test (MAST), CAGE, and the Lifetime Alcohol Consumption History were obtained for each alcoholic.

Major Findings:

The research design has been modified and improved from the one submitted last year. Data from the adult study have been collected and are being analyzed. The sources for the children's study have been identified.

Significance to Biomedical Research and the Program of the Institute:

Studies of underserved and relatively understudied minorities are important and emphasized. Comparative studies will enable a better understanding of the prevalence of comorbidity and possibly lead to improved treatment. Determination of risk and protective factors in children are of importance to improved prevention efforts.

Proposed Course:

The data will be submitted for publication.

Publications:

None.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00059-03 LCS

PERIOD COVERED

October 1, 1993 to September 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Determinants of Cognitive Dysfunctions in Neuropsychiatric Disorders

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: H. Weingartner Section Chief LCS, NIAAA

Others: M. Eckardt Senior Investigator LCS, NIAAA  
D. George Senior Clinical Investigator LCS, NIAAA  
D. Johnson IRTA Fellow LCS, NIAAA  
W. Lombardi IRTA Fellow LCS, NIAAA  
N. Ramsey Visiting Fellow LCS, NIAAA

COOPERATING UNITS (if any)

NIMH (S. Molchan, T. Sunderland); NINDS (J. Grafman); U of Toronto (C. Szostak); UCSF (O. Wolkowitz); SUNY (H. Begleiter); U of Haifa, Israel (S. Breznitz); NIA (P. Costa); U of Strausbourg, France (J. Danion); NIDA (J. Henningfield)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Cognitive Neuroscience

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Subgroups of alcoholics express selective impairments in cognitive functions that require reflection (monitoring and evaluative functions as well as operations that are under voluntary control that are used to allocate cognitive resources). In contrast, the elderly, and many types of neuropsychiatric disorder patients, are unimpaired in their ability to use reflective functions even when they demonstrate impairments in memory and attention. Drug and behavioral challenges are useful in delineating the types of cognitive impairments that are present in these different types of patients (i.e., a benzodiazepine challenge in detoxified alcoholics, stimulant and cholinergic drugs challenges in elderly normal volunteers and in Alzheimer's disease patients, ketamine in schizophrenic patients and normal controls, and DHEA administration in depression).

Project Description:Investigators:

H. Weingartner	Section Chief	LCS, NIAAA
H. Begleiter	Professor	SUNY
S. Breznitz	Professor	U of Haifa, Israel
P. Costa	Chief	LPC, NIA
J. Danion	Chair	Dept. of Psychology, U of Strausbourg, France
M. Eckardt	Senior Investigator	LCS, NIAAA
D. George	Senior Clinical Investigator	LCS, NIAAA
J. Grafman	Section Chief	CNS, NINDS
J. Henningfield	Chief	LCS, NIDA
D. Johnson	IRTA Fellow	LCS, NIAAA
W. Lombardi	IRTA Fellow	LCS, NIAAA
S. Molchan	Investigator	LCS, NIMH
N. Ramsey	Visiting Fellow	LCS, NIAAA
T. Sunderland	Section Chief	LCS, NIMH
C. Szostak	Professor	Dept. of Psychology, U of Toronto
O. Walkowitz	Professor	Dept. of Psychiatry, U Cal, San Francisco

Objectives:

The aim of this project is to discover mechanisms that would account for impairments in cognitive functioning in neuropsychiatric disorders, particularly in syndromes that are associated with alcohol abuse. Studies are designed to help define the distinctive mechanisms and cognitive operations that are used in reflective cognitive processes (processes that are under voluntary control and part of consciousness) as expressed in memory, attention, perception, and related mental functions in contrast to cognitive functions that are carried out automatically or those that are outside of awareness. Impairments in such reflective cognitive operations are hypothesized to be an important determinant (risk factor) for the development of alcoholism as well as maintaining alcohol abuse. Findings are used for the development of models of normal and impaired cognitive functioning, the development of better diagnostic procedures for identifying forms of cognitive impairment, and new treatment strategies for altering patterns of behavior that result in alcohol abuse.

Methods Employed:

Laboratory tests and procedures that assess different components of cognitive functions (types of memory, as well as attention, perception, olfaction, and related cognitive operations that are under voluntary control in contrast to operations that are carried out automatically or outside of awareness) are administered and related to measures of brain function including neuropathological findings, neurotransmitter metabolites, and patterns of brain activity as assessed by imaging and evoked response methods.

Major Findings:

Subgroups of alcoholics, as well as other populations of drug abusing patients, demonstrate highly selective impairments in reflective cognitive functions that are under voluntary control as evidenced by inability to: (1) suppress intrusions in memory, (2) accurately evaluate cognitive performance, (3) identify the source of remembered events, and (4) allocate cognitive resources and strategically shift resources, (i.e., process and recall not only self-generated events but also information that they are asked to remember). In contrast, normal controls, even the very elderly, are typically highly effective in

monitoring their own cognitive performance and therefore able to effectively, strategically, allocate cognitive resources. This is also the case in other populations of cognitively impaired neuropsychiatric disorder patients (i.e., clinically depressed patients). The impairments in reflective operations that are apparent in alcoholics are expressed in perception as well as in learning and remembering. These specific deficits in cognition are related to patterns of altered brain activity, i.e., alterations in glucose utilization in left prefrontal, temporal, and posterior orbital frontal cortical regions. Other populations of neuropsychiatric disorder patients, i.e., depressed patients, Alzheimer's patients, and amnesic disorder patients manifest a rather different form of cognitive impairment from those expressed in alcoholics. For example, memory impaired depressed patients demonstrate no impairment in reflective cognitive functions despite impaired learning and memory and their cognitive bias to undervalue their cognitive performance. In these depressed patients, basal serum levels of the adrenal steroid dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S) have been shown to be correlated with performance on tasks that are used to assess automatic cognitive operations and access to semantic knowledge. Drug and behavioral challenges further highlight the types of cognitive impairments that are present in these different populations of patients (i.e., a benzodiazepine challenge in detoxified alcoholics, stimulant and cholinergic drugs challenges in elderly normal volunteers and in Alzheimer's disease patients, response to different classes of olfactory stimuli in alcoholics, and ketamine in schizophrenic patients, DHEA administration in depression). This research is being extended to the study of alcohol craving and the study of children that are at risk for developing alcohol.

#### Significance to Biomedical Research and the Program of the Institute:

These findings and methods can be used to define how cognitive functions may fail in different populations of patients, particularly those that abuse alcohol. The results obtained are directly applicable in the diagnosis of neuropsychiatric disorders in general and disorders that are related to alcohol abuse in particular. Similarly, these findings are of value in evaluating the effectiveness of treatments designed to attenuate or reverse cognitive impairments. These results are also useful in the development of psychobiological models of memory.

#### Publications:

Johnson DN, Yantis S. Allocating visual attention: Tests of two process models, *Journal of Experimental Psychology: Human Perception and Performance*, in press.

Lombardi W, Weingartner HJ. Pharmacological treatment of impaired memory functions. In: Baddeley A, Wilson B. eds. *Handbook of Memory Disorders*. Jon Wiley Press, in press.

Szostak C, Lister R, Weingartner H. Dissociative effects of mood on access to memories in psychological concepts and dissociative disorders. In: Miller R, Doane B, eds. *Lawrence Erlbaum Associates, Inc.*, 1994;187-206.

Weingartner HJ, Eckardt M, Grafman J, Molchan S, Putnam K, Rawlings R, Sunderland T. The effects of repetition on memory performance in cognitively impaired patients, *Neuropsychology* 1993;7(3):385-95.

Weingartner HJ, Giambra L, Kawas C, Rawlings R, Shapiro M. Changes in Semantic Memory in Alzheimer's disease patients, *The Gerontologist* 1993;33(5):637-43.

Weingartner HJ, Hommer D, Molchan S, Robinson JK, Sunderland T. Conceptual and practical issues in the development and assessment of drugs that would enhance cognition. In: Herrmann, Johnson, Hertzog, Hertel, eds. *Basic and applied memory: Research on practical aspects of memory*. Lawrence Erlbaum Associates, Inc., in press.

Wolkowitz OM, Reus V, Manfredi F, Ingbar J, Brizendine L, Weingartner H. Ketoconazole administration in hypercortisolic depression, Am J Psychiatry, in press.

Wolkowitz OM, Weingartner HJ. Steroid modulation of human memory: Biochemical correlates, Biol Psychiatry 1993;33:744-46.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00060-03 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Drug Effects on Memory and Related Cognitive Functions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	H. Weingartner	Section Chief	LCS, NIAAA
Others:	M. Eckardt	Senior Investigator	LCS, NIAAA
	D. George	Senior Clinical Investigator	LCS, NIAAA
	D. Hommer	Section Chief	LCS, NIAAA
	D. Johnson	IRTA Fellow	LCS, NIAAA
	W. Lombardi	IRTA Fellow	LCS, NIAAA

COOPERATING UNITS (if any)

NIMH (S. Molchan, T. Sunderland, A. Brier, W. Potter); U Strasbourg (J. Danion); U British Columbia (E. Eich); U London (S. File, E. Joyce); Upjohn Corp. (J. Fleishaker); NIDA (J. Henningfield); U CA (O. Wolkowitz); E. Lilly (S. Paul)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Cognitive Neuroscience

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOXES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Acute administration of benzodiazepines in normal volunteers simulates the type of cognitive impairment (dysfunction in reflective (control) cognitive operations) found in detoxified alcoholics and potentiates that cognitive deficit in alcoholics. Normal volunteers, treated with the benzodiazepine triazolam, are unable to effectively, strategically, shift their attention, are not able to appreciate the extent to which they are sedated, cannot monitor the accuracy or the source of what they remember, and are unable to suppress errors in performing cognitive tasks despite the fact that many other facets of their cognitive functioning are spared. The effect of acute alcohol administration on cognition are similar to those expressed by the benzodiazepines and are different from other classes of drugs, i.e., cholinergic antagonists, but similar to the effects of the anesthetic ketamine, an antagonist of the NMDA-type glu receptor (a receptor believed to be involved in long-term potentiation (memory consolidation). Drugs such as benzodiazepines, and alcohol in normal controls, also produce qualitative shifts in how both normal controls and alcoholics retrieve previously acquired knowledge. That is, these types of drugs alter retrieval context which is manifest in the specific facets of previously acquired experiences that can be recalled. This is expressed in various forms such as state (context)-dependent retrieval of self generated information, memory for the source of knowledge, and evaluation of intrusion errors. Paradoxically, the presence of these drugs at the time of retrieval can enhance the amount of information that can be remembered for information acquired just prior to drug administration. The profile of highly specific effects of benzodiazepines (and alcohol) on reflective functions may be important in understanding patterns of uncontrolled drinking in alcoholics and also provides data on the stimulus discriminative (and reinforcing) properties of these drugs. This research is being extended to the study of alcohol craving and the study of children that are at risk for alcoholism.

Project Description:Investigators:

H. Weingartner	Section Chief	LCS, NIAAA
A. Brier	Senior Investigator	ETB, NIMH
J. Danion	Professor	U     Strausbourg, France
M. Eckardt	Senior Investigator	LCS, NIAAA
E. Eich	Professor	U     B r i t i s h Columbia, Canada
S. File	Professor	U London, UK
J. Fleishaker		Upjohn Corp.
D. George	Senior Clinical Investigator	LCS, NIAAA
J. Henningfield	Chief	CPRB, NIDA
D. Hommer	Section Chief	LCS, NIAAA
D. Johnson	IRTA Fellow	LCS, NIAAA
E. Joyce	Professor	U London, UK
W. Lombardi	IRTA Fellow	LCS, NIAAA
S. Molchan	Investigator	LCS, NIMH
S. Paul		Eli Lilly, Indianapolis, IN
W. Potter	Section Chief	LCS, NIMH
T. Sunderland	Section Chief	ETB, NIMH
C. Szostyk	Psychologist	U Toronto, Canada
O. Wolkowitz	Professor	U Cal, San Francisco

Objectives:

The broad objectives of this project are to: (1) understand changes in cognition that occur in response to alcohol and related psychoactive drugs (particularly those aspects of cognitive functioning that are under voluntary control in contrast to operations that are outside of awareness), and to use this information to: (2) define the mechanisms that mediate different types of memory and related cognitive functions; (3) develop models that would account for how cognition is impaired in neuropsychiatric disorders in general and in syndromes associated with alcohol abuse in particular; (4) understand factors that may account for uncontrolled drinking; and (5) to develop strategies for treating impairments in cognitive functioning.

Methods Employed:

Laboratory tests and procedures are used that assess different components of cognitive functions (types of memory, as well as attentional, perceptual, and related cognitive operations that are under voluntary control in contrast to operations that are carried out automatically or outside of awareness). These methods are used to evaluate how drugs alter cognition in unimpaired subjects as well as in cognitively impaired patients. Findings are related to measures of brain function including neuropathological findings, neurotransmitter metabolites, and patterns of brain activity as assessed by imaging and evoked response methods.

Major Findings:

Benzodiazepines impair the ability of subjects to use reflective (control) cognitive operations not only in explicit memory (as reported previously) but also in attention, perception, and related mental functions. Normal volunteers, treated with the benzodiazepine triazolam, are unable to effectively, strategically, shift their attention, are not able to appreciate the extent to which they are sedated, can not monitor the accuracy or the source of what they remember, and are unable to suppress errors in performing cognitive tasks despite the fact that many other facets of their cognitive functioning are spared.



Effects of other benzodiazepines, such as alprazolam and adinazolam, produce similar dose-dependent sedative and cognitive effects which simulate the cognitive deficits expressed in untreated amnesic patients. While similar effects are apparent in benzodiazepine-treated detoxified alcoholics, there are also some qualitative differences in the pattern of cognitive responses obtained in the alcoholic, suggesting that this type of drug challenge may prove to be useful for diagnostic purposes as well as for developing new types of therapeutics for the treatment of alcoholism. The effects of alcohol on cognition are similar to those expressed by the benzodiazepines and are different from other classes of drugs, i.e., cholinergic antagonists, but similar to the effects of the anesthetic ketamine, an antagonist of the NMDA-type glu receptor, a receptor believed to be involved in long-term potentiation (memory consolidation). Recent studies have also demonstrated that when subjects acquire knowledge just prior to the administration of a benzodiazepine such as triazolam, later recall of that information is, paradoxically, enhanced. This effect has been shown to not be due to a drug-induced blockade of memory interference effect but rather to qualitative changes in how subjects retrieve information from memory. This research has also led to the development of studies designed to investigate benzodiazepine and alcohol effects on memory retrieval (state (context)-dependent remembering) in both normal controls as well as in detoxified alcoholics (treated with benzodiazepine but not alcohol). The profile of highly specific effects of benzodiazepines (and alcohol) on reflective functions is important for understanding patterns of uncontrolled drinking in these patients and also sheds some light on the stimulus discriminative (and reinforcing) properties of these drugs in terms of the selective aspects of autobiographical memory that are elicited under drug, in contrast to undrugged conditions. The specific types of cognitive changes that are induced by benzodiazepines (and alcohol) are currently being developed to provide us with: (1) models of the cognitive changes apparent not only in the amnesic alcoholic, but also in nominally cognitively unimpaired detoxified alcoholics, and (2) knowledge about the determinants of the cognitive changes, particularly those that involve reflective (control) functions, should prove useful for the development of more effective treatments for alcoholism.

#### Significance to Biomedical Research and the Program of the Institute:

These findings are important in understanding the mechanisms that account for normal memory as well as forms of impaired memory functions. This research also serves as a basis for the development of drug and behavioral treatments of cognitively impaired patients and addictive behavior.

#### Publications:

Danion JM, Weingartner HJ, File SE, Jaffard R, Sunderland T, Tulving E, Warburton DM. Pharmacology of human memory and cognition, *J Psychopharmacol* 1993;7(4):371-7.

Fleishaker JC, Garzone PD, Chambers JH, Sirroco K, Weingartner HJ. Comparison of the psychomotor and memory effects of adinazolam and alprazolam after single doses in healthy normal volunteers, *J Clin Psychopharmacol*, in press.

Hommer D, Weingartner HJ, and Brier A. Dissociation of benzodiazepine induced amnesia form sedation, *Psychopharmacology* 1993;112:455-60.

Sunderland T, Molchan S, Martinez R, Vitiello B, Putnam, K, Martin A, Weingartner H. Functional cholinergic receptor sensitivity: The role of drug probes. *Pharmacological Basis of Cholinergic Therapy in Alzheimer's Disease*, in press.

Weingartner H. Evaluating the cognitive response to drug treatments in Cognitive Function Measures: Applications in patient studies. In: Eldin M, Fleishaker J, Garzone P, eds. *Pharmacodynamics and Pharmacokinetics vol. 4, Drug Treatments of Memory Impairments*. Cincinnati, Ohio, 1993.

Weingartner HJ, Crawley J, Hommer D, Molchan S, Raskin A, Robinson A, Sunderland T. Practical and conceptual issues in the development of drugs for enhancing cognition. In: Herrmann, Johnson, Hertzog, Hertel, eds. Basic and Applied Memory: Research on Practical Aspects of Memory: Lawrence Erlbaum Associates, Inc., in press.

Weingartner HJ, Joyce EM, Adams CM, Eckardt HJ, George T, Lister RG. Effects of the benzodiazepine triazolam on different forms of memory and sedation, J Psychopharmacol 1993;7(4):305-15.

Weingartner HJ, Sirocco K, Rawlings R, Joyce E, Hommer D. Dissociations in the expression of the sedative effects of triazolam, Psychopharmacology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00061-03 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Cerebral Metabolic Correlates of Aggressive and Addictive Behavior**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. Andreason	Senior Clinical Investigator	LCS, NIAAA
Others:	G. Brown	Clinical Director	LCS, NIAAA
	M. Eckardt	Senior Investigator	LCS, NIAAA
	D. George	Senior Clinical Investigator	LCS, NIAAA
	D. Hommer	Section Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA
	D. Rio	Physicist	LCS, NIAAA
	U. Ruttimann	Biomedical Engineer	LCS, NIAAA

COOPERATING UNITS (if any)

Nuclear Medicine Department, NIHCC (P. Herscovitch)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Brain Electrophysiology and Imaging

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This research is designed to determine neuroanatomical and neurochemical correlates of addictive and aggressive/impulsive behavior in human subjects. The principal focus of these studies is the measurement and correlation of regional cerebral glucose metabolic activity, using positron emission tomography (PET), cerebrospinal fluid metabolites, and measures of impulsive/aggressive behavior and excessive alcohol consumption.

Past studies have correlated decreasing cerebrospinal fluid (CSF) levels of 5-hydroxy-indoleacetic acid (5-HIAA) with increasing lifetime histories of aggression. We demonstrated that decreased rCMRglu in the orbital-frontal and right temporal regions of the brain is associated with increased lifetime history of aggression. We have also shown differences in orbital-frontal glucose metabolism between men and women; similar gender-related differences in CSF 5-HIAA have been documented. The intent of the present project is to correlate CSF 5-HIAA levels and rCMRglu in the orbital-frontal and right temporal regions with impulsive/aggressive behavior. Subject accrual continues. Scan data comparing alcoholics and normal volunteers are under analysis. When a statistically significant number of research subjects have completed the study, CSF samples will be analyzed in one batch.

Project Description:Investigators:

P. Andreason	Senior Clinical Investigator	LCS, NIAAA
G. Brown	Clinical Director	LCS, NIAAA
M. Eckardt	Senior Investigator	LCS, NIAAA
D. George	Senior Clinical Investigator	LCS, NIAAA
D. Hommer	Section Chief	LCS, NIAAA
P. Herscovitch	Section Chief	NMDCC, NIH
M. Linnoila	Scientific Director	NIAAA
D. Rio	Physicist	LCS, NIAAA
U. Ruttimann	Biomedical Engineer	LCS, NIAAA

Objectives:

The objective of this research is to study the neuroanatomical and neurochemical correlates to addictive and aggressive/impulsive behavior in human subjects. A primary focus of these studies is the measurement and correlation of regional cerebral glucose metabolic activity using positron emission tomography (PET), and cerebrospinal fluid metabolites with measures of impulsive/aggressive behavior and alcohol consumption.

Pharmacologically-induced changes in aggressive/impulsive behavior on rCMRglu will be determined. Lithium carbonate has been shown to decrease "rule-breaking" in samples of prison inmates in double-blinded, placebo-controlled studies. For this reason, a selected group of patients and volunteers will undergo treatment with lithium carbonate to reach a serum lithium level of 0.8 meq/l. This will be maintained for three months, following which, the patients will undergo repeat PET scanning, lumbar puncture (LP), and clinical ratings for aggression. This will not only allow us to see the effects of lithium carbonate on brain metabolism, but will help correlate changes in rCMRglu with changes in aggressive behavior.

Methods Employed:

Measurements of rCMRglu are made via the 2-deoxyglucose method using <sup>18</sup>F-2-fluoro-2-deoxyglucose as a PET tracer. PET scanning will be done on the Scanditronix 2048-15B camera in the Nuclear Medicine Department of the Clinical Center of NIH. This scanner provides 5 mm in-plane and 5 mm z-axis resolution. Patients and controls will also have high resolution MRI of the brain performed for volumetric analysis of brain structure and MRI-PET co-registration. This will provide more precise anatomical definition of regional metabolic activity. MRI scans will be performed on the Sigma 1.5 tesla MRI scanner in the *in vivo* MRI research center at the NIHCC. Scans will be analyzed by both region of interest analysis and pixel by pixel subtraction of averaged group scans. Regression analysis will correlate scores from the Brown-Goodwin Life-time History of Aggression Scale and Hostility and Direction of Hostility Questionnaire with orbital-frontal and right temporal regions of interest and CSF 5-HIAA.

CSF analysis will be performed on patients and volunteers on the 3BN patient care unit at NIHCC. All subjects will be maintained on a low monoamine diet at least three days prior to the LP and PET scan as CSF 5-HIAA levels change with dietary tryptamine intake. Clinical CSF fractions will be analyzed by the central labs for clinical care purposes. Research CSF fractions will be frozen and analyzed by the NIAAA research labs.



Major Findings:PET rCMRglu findings in personality-disordered patients with aggressive/impulsive behavior

This study was conducted in collaboration with P. Goyer and R. Cohen while at the Section on Clinical Brain Imaging, LCM, NIHM. In this study, patients with symptoms of increased impulsive/aggressive behavior were scanned using FDG-PET. Patients were also rated with the Brown-Goodwin Life-time History of Aggression Scale. These patients did not meet criteria for drug or alcohol addiction or any axis I DSM-III-R psychiatric disorder. These patients met criteria for various axis II DSM-III-R personality disorders (PD) including antisocial PD, borderline PD, dependent PD, and PD not otherwise specified. It was found that decreasing orbital-frontal and right temporal glucose metabolic rates correlated with increasing life-time history of aggression regardless of the PD diagnosis.

Gender-related rCMRglu differences as measured by PET

This study was conducted in collaboration with A. Zametkin and R. Cohen while at the Section of Clinical Brain Imaging, LCM, NIHM. In this study, male and female normal volunteers were scanned as controls for several clinical projects. Few high resolution PET data on gender-related rCMRglu differences existed. It was found that orbital-frontal rCMRglu was significantly higher in women than in men as was global gray glucose metabolism. This PET finding was hypothesized to be related to findings that women have higher CSF 5-HIAA levels than men.

Significance to Biomedical Research and the Program of the Institute:

These studies have strongly suggested that the orbital-frontal regions of the brain are connected to impulse control. Orbital-frontal glucose metabolism may be a biological marker that correlates with subjects' ability to control impulsive/aggressive behavior. Since these behaviors also correlated to serotonin activity (CSF 5-HIAA), these findings suggest that medications that increase serotonin activity in the orbital-frontal regions may decrease impulsive/aggressive behavior.

Publications:

Andreason PJ, Zametkin A, Guo A, Baldwin P, Cohen RM. Gender-related PET differences in normal controls, *Psychiatry Res* 1994;51:175-83.

Goyer PF, Andreason PJ, Semple WE, Clayton AH, King AC, Compton-Toth BA, Schultz SC, Cohen RM. Positron-emission tomography and personality disorders, *Neuropsychopharmacology* 1994;10:21-8.

Rumsey JM, Zametkin A, Andreason PJ, Hanahan AP, Hamburger SD, Aquino T, King AC, Pikus A, Cohen RM. Normal activation of frontotemporal language cortex in dyslexia, as measured with 0-15 positron emission tomography, *Arch Neurol* 1994;51:27-38.

Rumsey JM, Andreason PJ, Zametkin A, Aquino T, King AC, Hamburger S, Pikus A, Rappaport JL, Cohen RM. Failure to activate left temporoparietal cortex in dyslexia: A 150 PET study, *Arch Neurol* 1992;49:527-34.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00062-03 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Brain Serotonin Synthesis in Patients with Addictive and Aggressive Behaviors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. Andreason	Senior Clinical Investigator	LCS, NIAAA
Others:	G. Brown	Section Chief	LCS, NIAAA
	M. Eckardt	Senior Investigator	LCS, NIAAA
	D. George	Senior Clinical Investigator	LCS, NIAAA
	D. Hommer	Section Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA
	D. Rio	Physicist	LCS, NIAAA
	S. Shoaf	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS (if any)

Nuclear Medicine Department, NIHCC (W. Eckelman, B. Schmall); Dept Med, U Brit Columbia (D. Doudet)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section on Brain Electrophysiology and Imaging

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project is designed to develop and implement a novel positron emitting tracer 11-C-alpha-methyl-tryptophan (CAMT) in order to measure the local distribution and accumulation of brain serotonin in patients with alcoholism, varying degrees of aggressive/impulsive behavior, and normal volunteers.

CAMT is currently not approved for human use in the United States. In order to develop CAMT for humans, we must provide toxicology and dosimetry data that fulfill Food and Drug Administration (FDA) and Radiation Safety Committee guidelines, gather kinetic data in higher animals, and modify the synthesis to produce the L-form of CAMT.

The Request for Proposal (RFP) will be released in the Commerce Business Daily in early fall. The RFP calls for completion of the project within four months after award of contract. This will give toxicology information necessary for FDA approval of alpha-methyl-tryptophan in humans. Concomitantly, Dr. Schmall will synthesize CAMT to measure organ dosimetry in Rhesus macaques. After these data are acquired, application for use of CAMT in humans will be submitted to the FDA.

Dr. Shoaf is determining pharmacokinetic, distribution, and enzyme kinetic properties in Rhesus macaques so that these data may be used to calculate values for serotonin synthesis in both monkeys and humans.

Project Description:Investigators:

P. Andreason	Senior Clinical Investigator	LCS, NIAAA
G. Brown	Clinical Director	LCS, NIAAA
D. Doudet	Assistant Professor	U Brit Columbia, Canada
M. Eckardt	Senior Investigator	LCS, NIAAA
W. Eckelman	Section Chief	NMDCC, NIH
D. George	Senior Clinical Investigator	LCS, NIAAA
S. Goodson	Senior Staff Fellow	LCS, NIAAA
D. Hommer	Section Chief	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
D. Rio	Physicist	LCS, NIAAA
U. Ruttimann	Biomedical Engineer	LCS, NIAAA
B. Schmall	Research Chemist	NMDCC, NIH
S. Shoaf	Senior Staff Fellow	LCS, NIAAA

Objectives:

The aim of this research is to determine the neuroanatomical distribution of serotonin activity in patients with addictive and aggressive/impulsive behavior. This will be accomplished by developing and implementing a novel positron emitting tracer <sup>12</sup>C-alpha-methyl-tryptophan (CAMT) to measure the differential distribution and accumulation of brain serotonin in patients with alcoholism, varying degrees of aggressive/impulsive behavior, and normal volunteers. In order to develop CAMT for human use, we must provide toxicology and dosimetry data that fulfill Food and Drug Administration and Radiation Safety Committee guidelines, gather kinetic data in higher animals to confirm that the lumped rate constant does not vary significantly across species, and modify the synthesis to produce the L-form of CAMT.

Methods Employed:

CAMT is a substrate for the enzymes in the serotonin synthetic pathway. CAMT is taken up by serotonin neurons via the amino-acid transport system and converted to alpha-methyl-serotonin (AMS) by the action of tryptophan hydroxylase and aromatic amino-acid decarboxylase. AMS is accumulated preferentially in the presynaptic terminals of serotonin neurons. When it is released by the presynaptic terminal, it is either taken up by presynaptic terminals or slowly diffuses away. It is not a substrate for monoamine oxidase and, therefore, is not metabolized. This property makes CAMT uniquely suited for noninvasively imaging presynaptic serotonin activity in the human brain via positron emission tomography.

CAMT is currently not approved for human use in the United States. Kinetic studies of CAMT have only been published for rodents. Dosimetry data for CAMT have been acquired in Canada but not yet published. Published synthesis methods for CAMT produce a racemic mixture while the enzymatic substrate is the L-form. A combined rate constant (or 'lumped constant') must be known in order to calculate the rate of formation of serotonin using CAMT; this has been measured in rats, but it has yet to be confirmed that this rate constant is the same across species.

(1) Synthesis of stereospecific L-CAMT. Although the original synthesis method as described by M. Dicksic at McGill University was reported to be stereospecific, other laboratories have only been able to produce the racemic compound. L-CAMT will provide the highest specific activity (signal-to-noise ratio). D-CAMT would not be taken up by the serotonin neurons and would be imaged along with tissue-free L-CAMT. L-CAMT will be obtained from the racemic mixture via a chiral column.

(2) Toxicology studies. Acute toxicology and pathology studies are underway. Acute dosing, sacrifice of animals, and gross necropsy is complete. No animals were lost in either the AMT or placebo groups of either mice or rabbits. Gross necropsy shows no difference between the placebo or AMT groups. Histopathological review is underway, but results are not complete.

(3) Determination of pharmacokinetic, tissue distribution, and enzyme kinetic differences. A combined rate constant (or "lumped constant") must be determined in order to calculate the rate of formation of serotonin using CAMT. Three variables which must be measured for the calculation of the lump constant are the pharmacokinetic, tissue distribution, and enzyme kinetic differences between tryptophan and CAMT. We will use monkeys to determine these differences in lieu of human subjects as these differences cannot be determined without destructive sampling. Dr. Shoaf and her laboratory will write the protocol and perform these animal studies.

(4) Radiation dosimetry data. Rat dosimetry data will be provided by M. Dicksic at McGill University and will be used to calculate a millicurie/kg dose that will be within the safety guidelines of the Radiation Safety Committee. Two monkeys will then receive this dose and undergo total-body scanning on the Posicam scanner in the NMDCC. Raw count data will be used to calculate radiation dosimetry that will be more representative of human subjects.

#### Significance to Biomedical Research and the Program of the Institute:

CAMT PET scanning will provide a means to measure the contributions of serotonin synthesis and storage to addictive and aggressive/impulsive behavior in humans to be studied. Past work has linked serotonin to aggressive/impulsive and addictive behaviors, but the techniques used to measure serotonin activity in past *in vivo* studies have been indirect. CAMT PET scanning, once developed, will provide a noninvasive, direct technique to measure serotonin synthesis and storage in the human brain.

#### Major Findings:

Total arterial tryptophan (TP) concentrations in normal rhesus are  $9.2 \pm$  mg/ml. Free TP concentrations are  $2.5 \pm 0.6$  mg/ml or approximately 26%.

At concentrations of 500 ng or less, alpha-methylTP is 98% unbound.

Following a bolus dose of 50 mcg/kg of alpha-methylTP to four anesthetized monkeys that were observed over a four hour period, the kinetics of alpha-methylTP, were best described by a 3 compartment open model.

#### Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

201 AA 00250-11 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Electrophysiological Studies of Acute and Chronic Alcohol Consumption**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Eckardt Senior Investigator LCS, NIAAA

Others: C. Adams IRTA Fellow LCS, NIAAA  
M. Linnoila Scientific Director NIAAA  
D. Rio Physicist LCS, NIAAA

COOPERATING UNITS (if any)

Department of Psychiatry, Washington University (J. Rohrbaugh); Department of Psychology, Catholic University (R. Parasuraman); Department of Electrical Engineering, University of Nebraska (J. Varner)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Brain Electrophysiology and Imaging

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The aim of this study is to detect covert brain processes that underlie cognition and performance in human subjects, and the acute and chronic effects of ethanol upon such processes. Included is an extensive study in which we are examining brain processes in individuals with different family histories of alcoholism. A principal focus of these studies is the measurement of brain electrical potentials, which provide information regarding the timing and character of the constituent sensory, cognitive, and motor elements that are the mechanisms underlying observable behavior. The study of the brain potentials also allows inference of the specific brain regions affected by alcohol. The brain electrical potentials are studied within a broad context provided by performance and psychometric data, and measurement within other psychophysiological response systems.

We have obtained data which document a large number of acute and chronic effects of alcohol on specific brain functions, ranging from sensory input to motor control functions. Of particular interest is a finding that brain electrical and autonomic signs of alerting and orienting are enhanced by alcohol, in contrast to its depressant effect on most other functions. A similar effect was observed in a sample of chronic alcoholic, organic brain disorder patients. Such findings suggest that alcohol intoxication and alcoholic organic brain disease may be associated with a disinhibition or deregulation of orienting processes.



Project Description:Investigators:

M. Eckardt	Senior Investigator	LCS, NIAAA
C. Adams	IRTA Fellow	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
R. Parasuraman	Professor	Catholic U, Washington, DC
D. Rio	Physicist	LCS, NIAAA
J. Rohrbaugh	Associate Professor	Washington U, St. Louis, MO
J. Varner	Associate Professor	U of Nebraska Omaha, NE

Objectives:

This research aims to provide a comprehensive overview of the effects of alcohol, both acute and chronic, on sensory, cognitive, and motor systems. A primary focus is on event-related brain electrical potentials (ERPs), elicited in response to environmental stimulation, and extracted by computer from the on-going EEG. The electrical responses are studied in various tasks and under various conditions for information about the contributory neural processes and possible disturbances associated with alcohol consumption. Measurement of the brain electrical potentials is accompanied by simultaneous measurements of psychophysical judgments and autonomic and somatic system responsivity.

Methods Employed:

Brain electrical activity and other psychophysiological responses are measured from surface electrodes using conventional EEG and polygraph instruments, allowing data to be acquired simultaneously from as many as 40 channels. The responses are analyzed with respect to wave form and sensitivity to experimental variables using multivariate techniques. Topographic distributions of the responses over the scalp are studied for evidence of neural sources of the electrical activity, using scalp mapping and dipole inference techniques.

Sensory functions are evaluated separately for visual, acoustic, and somatosensory systems using clinically validated techniques. Visual stimulation and recording techniques permit evaluation of function in retina, optic nerve and tract, and cortical centers. Similarly, auditory and somatosensory techniques permit examination of function in peripheral, brainstem, and cortical areas. Cognitive function is assessed by examining responses that are related to attention and judgment using paradigms in which information content and relevance are manipulated.

Noteworthy progress has been made on two methodological problems. One is the development of methods for objective classification of EEG phenotypes using a parametric method of estimating frequency composition based on autoregressive filtering. This method is currently being expanded to allow for assessment of both temporal and spatial EEG nonstationarities, for possible genetic analysis. A second area of notable progress is in the installation and application of computer software for analysis of electromotive sources of brain electrical signals. This software is being elaborated to provide objective criteria for estimation of the number of contributory sources.

Major Findings:

(1) Genetics of human EEG and ERPs. This study is conducted with Dr. D. Goldman from the Laboratory of Neurogenetics and has entailed the acquisition of EEG and ERP signals under various conditions from a preliminary sample of more than 160 individuals drawn from more than 40 different families. In accord with previous



findings, a number of distinctive EEG phenotypes have been identified and distributions of these phenotypes in other family members are being studied. A number of families have been identified in which specific phenotypes appear to be segregating. In addition, we are proceeding with the development of alternative schematas for the identification and classification of EEG and EEG phenotypes, including the objective method described above.

(2) ERP signs of orienting in alcoholic organic brain syndromes (OBS). In a study of the O wave to infrequent visual and auditory stimuli, it was found that the O wave was markedly enhanced in groups of patients diagnosed with Korsakoff's syndrome and alcoholic dementia, in comparison to a group of age-matched normals. A simple measure based on O wave amplitude correctly classified more than 90% of the normal and patient subjects. The enlarged O wave in the patients was accompanied by exaggerated heart rate responses, particularly the acceleration component peaking at about two seconds post stimulus. The O wave enlargement is similar, although of less extent, to that previously observed by us in normal subjects during light sleep. These data are interpreted in terms of inadequate control over the orienting response, perhaps reflecting a frontal lobe deficit. Consistent with this hypothesis, the O wave amplitude is highly correlated with performance scores on standardized neuropsychological tests of frontal lobe function. In addition, it has been determined that O wave amplitude is highly correlated with memory performance. The relationship with memory performance appears to be specific to the alcoholic OBS patients, since no O wave enhancement has been obtained in a recently begun study of Alzheimer's dementia patients.

(3) Relationship of EEG to Positron Emission Tomography (PET) measures in alcoholic chronic OBS. A PET study of alcoholic OBS patients has revealed a number of subtle differences between patients and age-matched normal subjects. We have recently completed an analysis of the EEG data obtained simultaneously during the 40-minute isotope uptake period. Whereas spectral estimates of EEG frequency and amplitude are not greatly different if averaged over the entire 40-minute period, analysis of shorter epochs within the period reveals significant differences between normals and OBS patients. The normal subjects show a pronounced depression of EEG amplitude during a ten minute epoch immediately following injection. This transient depression is not apparent in the records from OBS patients. These EEG differences suggest the presence of differences in global response to the PET injection procedures, which may contribute to the observed PET differences in cerebral glucose utilization.

(4) ERPs in normal and in Attention Deficit/Hyperactivity Disorder children. An experimental protocol has been developed to provide a battery of behavioral and electrophysiological measures of attentional performance in children. An initial finding with a group of normal children indicates that the O wave in children is substantially larger than in adults.

#### Significance to Biomedical Research and the Program of the Institute:

Measurement of brain electrical activity permits studying information-processing mechanisms that occur in milliseconds and are outside of awareness. Moreover, distributional patterns of electrical activity enables localization. In that the acute and chronic effects of alcohol are known to alter brain electrical activity, EEG and ERP techniques afford unique research opportunities to study mechanisms of dysfunction and remediation.

#### Proposed Course:

Data will continue to be collected and analyzed.

#### Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00267-09 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Brain Imaging in Alcoholics with Organic Brain Syndromes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Eckardt Senior Investigator LCS, NIAAA

Others: M. Linnoila Scientific Director NIAAA  
R. Rawlings Mathematical Statistician LCS, NIAAA  
D. Rio Physicist LCS, NIAAA  
U. Ruttimann Biomedical Engineer LCS, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Brain Electrophysiology and Imaging

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Various in vivo imaging methods are being used to study the brains of alcoholics with organic brain syndromes. These techniques enable comparisons of gross anatomy (CAT - Computed Axial Tomography; MRI - Magnetic Resonance Imaging) of the brain with electrical activity (EEG - electroencephalography; ERPs - Event-Related Potentials) and rate of glucose utilization in specific regions (PET - Positron Emission Tomography). From a clinical perspective, these techniques, in association with other diagnostic tests, enable qualitative judgments to be made as to the anatomic and physiologic integrity of the brain.

Project Description:Investigators:

M. Eckardt	Senior Investigator	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
R. Rawlings	Mathematical Statistician	LCS, NIAAA
D. Rio	Physicist	LCS, NIAAA
U. Ruttimann	Biomedical Engineer	LCS, NIAAA

Objectives:

Our goals are to determine the structural-functional relationships in the brains of alcoholics with organic brain syndromes and compare them to those observed in age-matched normal volunteers.

Methods Employed:

Carefully selected samples of patients with alcohol amnesic disorder, commonly called Korsokoff's psychosis, and dementia associated with alcoholism have been studied with PET, CAT and/or MRI, and the electrical activity of the brain determined; these values are being compared to similar parameters obtained in age-matched normal volunteers.

All data obtained from the scanners are processed and displayed on our own image-processing system enabling us to analyze basic pixel data instead of post-processed images. Individual brain slices are oriented in three dimensions and displayed as stacked three-dimensional data or as surface contours. Using these methods, it is possible to display and calculate regional volumes and superimpose data from CAT, PET, and MRI, as well as other sources. This will insure that a regional alternation in glucose utilization corresponds to a particular anatomic location.

A critical review of the raw and reconstructed data obtained from PET, CAT, and MRI is carried out to insure that sources of noise inherent in each technique are taken into account, thereby insuring that artifacts will be correctly eliminated and confidence intervals may be more accurately estimated, leaving only statistically significant differences.

Resulting data are analyzed with mathematical techniques used in image processing, pattern recognition, and spectral analysis, i.e., by representing the spatial data in frequency space. Rigorous statistical tests are then used to study differences between various clinical populations.

Procedures have been developed to calculate and display scalp-monitored electrical potential fields and to estimate the position and distribution of electrical sources in the brain producing these potentials. This enables us to correlate anatomic or metabolic changes in the brain with modifications of cognitive processes as represented by changes in evoked responses.

Major Findings:

Localised cerebral glucose utilization was determined for nine abstinent alcoholic male patients with Korsakoff's syndrome and for 10 age-matched normal male volunteers using [<sup>18</sup>F]2-fluoro-2-deoxyglucose (FDG) positron emission tomography. Patients with Korsakoff's syndrome showed relatively decreased

utilization in cingulate and precuneate areas. These decreases persisted even when corrected for group differences in sulcal CSF measured on CT scans. EEG recordings before and during FDG uptake did not differ between the groups suggesting that these metabolic differences could not be explained by differential changes in physiological arousal at the time of scanning. It is concluded that these findings reflect a disruption of memory circuitry, Papez circuit, caused by diencephalic lesions induced by thiamine deficiency.

#### Significance to Biomedical Research and the Program of the Institute:

Establishing structure and function relationships among various areas of the brain is a crucial step in determining mechanisms. The approach advocated in the research described herein emphasizes: (1) detailed and intensive assessment of relatively few, carefully selected patients, thereby reducing heterogeneity in patient characteristics and enabling a convergence of information; and (2) comparing three-dimensional PET, CAT, and MRI images with each other and with electrical sources derived from scalp-monitored EEG and ERPs. Such studies have yet to be reported in the literature. Successfully combining these techniques will be a significant accomplishment with obvious applicability to other studies of brain structure and function.

#### Proposed Course:

Data collection and analyses have been completed and manuscripts are now being written.

#### Publications:

Joyce EM, Rio DE, Ruttiman UE, Rohrbaugh JW, Martin PR, Rawlings RR, Eckardt MJ. Decreased cingulate and precuneate glucose utilization in alcoholic Korsakoff syndrome, Psychiatry Research: Neuroimaging, in press.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00002-02 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Eye Movements in Alcoholism and Individuals at Risk for Alcoholism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. Hommer Section Chief LCS, NIAAA

Others: M. Israel Psychologist LCS, NIAAA

COOPERATING UNITS (if any)

Experimental Therapeutics Branch, NIMH (R. Litman); Child Psychiatry Branch, NIMH (J. Rapoport); Dept. of Psychiatry, Seattle VAMC (A. Radant)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Brain Electrophysiology and Imaging

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.4

PROFESSIONAL:

0.3

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The study of human eye movements provides an extremely useful approach to the examination of a variety of cognitive functions. It is obvious that the latency and goal saccadic eye movements are related to attention. What is not so obvious is that other aspects of cognition such as short-term memory, preparatory set, and inhibition of context inappropriate responses can also be assessed using eye movement techniques. Short-term memory, preparatory set, and inhibition of context inappropriate responses constitute core functions of the prefrontal cortex, the brain region most involved in the control of higher order cognitive processes. We have used a number of different tasks to elicit saccades. Primary among these have been a variety of delayed response tasks. These tasks allow us to independently assess core functions of the prefrontal cortex by measuring the accuracy and latency of memory guided saccades, as well as the frequency of context inappropriate saccades that interrupt the delay period. Using these tasks we have demonstrated that schizophrenics are impaired in all three core aspects of prefrontal cortex function while children with Attention Deficit Hyperactivity Disorder are impaired in only their ability to inhibit context inappropriate saccades. Studies of alcoholics and children at risk for alcoholism are planned.

Project Description:Investigators:

D. Hommer	Section Chief	LCS, NIAAA
M. Israel	Psychologist	LCS, NIAAA
R. Litman	Senior Clinical Investigator	ETB, NIMH
A. Radant	Psychiatrist	VAMC, Seattle
J. Rapoport	Branch Chief	CPB, NIMH

Objectives:

The purpose of this project is to characterize the saccadic and smooth pursuit eye movements of alcoholics and children at risk for the development of alcoholism. Our hypothesis is that eye movements that are dependent on function of the prefrontal cortex and associated basal ganglia thalamocortical circuits will be selectively impaired while those types of eye movements that are not dependent on prefrontal cortex will be normal in alcoholics and children at risk. Eye movements dependent upon the prefrontal cortex are those eye movements that must be triggered, guided, or inhibited on the basis of an internal representation of target position in time and space. Eye movements that can be programmed entirely on the basis of external visual stimuli do not require the prefrontal cortex for their accurate execution and should be normal in alcoholics and children at risk for the development of alcoholism. We plan to examine predictive and visually guided smooth pursuit eye movement as well as a variety of internally and externally guided saccades. In order to determine if children at risk for alcoholism show abnormal eye movement we will also need to determine the sequence of normal development for each of the various types of eye movements studied and to examine eye movements in children with Attention Deficit Hyperactivity Disorder (ADHD).

Methods Employed:

Eye movements will be measured using infra-red reflection oculography. Eye position data are recorded at a rate of at least 400 hz. and stored digitally for subsequent analysis. The data are analyzed using a group of computer algorithms which automatically divide the eye/target position record into a series of short segments that can be characterized as saccades, smooth pursuit, fixation, or artifact. These segments are further characterized in terms of their amplitude, velocity, peak velocity, initial and final distance from target, as well as their function (e.g., corrective saccade, intrusive saccade, square wave jerk, etc.). Targets are displayed on a video monitor in an otherwise completely dark room. In this way we can be sure that only the target or an internal representation of the target are available to guide eye movements.

Major Findings:

Most of the time spent on this project this year has involved software development required for the recording of eye movements. However, several new findings have emerged.

Children with ADHD showed a failure to inhibit inappropriate saccades during a Go-No task. They also showed a failure of inhibition during an oculomotor delayed response task. Other aspects of oculomotor function such as smooth pursuit speed and accuracy as well as saccadic peak velocity, accuracy, and latency are intact. These findings of a selective impairment of inhibition are very different from a more global pattern of eye movement dysfunction we have found among children with schizophrenia.

Significance to Biomedical Research and the Program of the Institute:

Since there is considerable knowledge regarding the functional neuroanatomy of the types of eye movements being studied, it will be possible to place any abnormalities of oculomotor control found in either alcoholism or among children at risk for alcoholism within a larger neurocognitive framework. Furthermore, since versions of all the eye movement tasks employed in this project have already been implemented in nonhuman primates, it may be possible to develop animal models for human eye movement dysfunctions in alcoholism and at risk for alcoholism. Finally, eye movement dysfunctions may be useful as genetic markers.

Proposed Course:

After initial studies among alcoholics and children at risk for the development of alcoholism are completed, we plan to combine eye movement recording with electroencephalographic techniques. We plan to use Laplacian analysis combined with dense electrode arrays to compare the scalp current distribution over the frontal and parietal lobes preceding memory guided and visually guided saccades. Through these approaches, we hope to confirm what has been suggested on the basis of lesion studies in non-human primates, namely that memory guided saccades require greater activation of prefrontal cortex than visually guided saccades. We also plan to use  $^{18}\text{O}$  PET and functional MRI techniques to characterize the distribution of cerebral blood flow during eye movement tasks thought to be dependent on prefrontal cortex activity.

Publications:

Cowley DS, Roy-Byrne PP, Radant A, Hommer DW, Greenblatt DJ, Vitaliano PP, Gordon C. Eye movement effects of diazepam in sons of alcoholic fathers and male control subjects, *Alcohol Clin Exp Res* 1994;18:324-32.

Litman RE, Hommer DW, Radant A, Clem T, Pickar D. Quantitative effects of typical and atypical neuroleptics on smooth pursuit eye tracking in schizophrenia, *Schizophrenia Research* 1994;12:107-20.

Ross RG, Radant AD, Hommer DW. A development study of smooth pursuit eye movements in normal children from 8 to 15 years, *J Am Acad Child Adolesc Psychiatry* 1993;32:783-91.

Ross RG, Radant AD, Hommer DW. Open- and closed-loop smooth pursuit eye movements in normal children: An analysis of a step-ramp task, *Dev Neuropsychology*, in press.

Ross RG, Radant AD, Hommer DW. Saccadic eye movements in normal children from 8 to 15 years of age: A developmental study of visuo-spatial attention, *Journal of Autism and Developmental Disorders*, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AA 00081-01 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Functional Magnetic Resonance Imaging of Olfactory Stimulus Processing**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. Hommer	Section Chief	LCS, NIAAA
Others:	N. Ramsey	Visiting Fellow	LCS, NIAAA
	R. Rawlings	Mathematical Statistician	LCS, NIAAA
	D. Rio	Physicist	LCS, NIAAA
	U. Ruttimann	Biomedical Engineer	LCS, NIAAA

COOPERATING UNITS (if any)

In Vivo NMR Center, NCRR (P. Van Gelderen, C. Moonen); LDRR (S. Duyn)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Electrophysiology and Brain Imaging

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects    ☐ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Unlike visual or tactile perception, the functional anatomy of odor perception in humans has received very limited attention. This is unfortunate because the brain regions involved in odor perception appear to overlap with the brain regions involved in motivation and emotion. Since, in alcoholics, states of craving for alcohol can be induced by the odor of alcoholic beverages and these states involve both motivational and emotional components, we felt, as a prelude to studies of the functional neuroanatomy of alcohol craving, it would be important to develop techniques to examine brain changes associated with olfactory perception.

Normal volunteers were exposed to various odorants using a continuous airflow system, while lying in a standard 1.5 Tesla MRI scanner. A pulse sequence developed at the In Vivo NMR Center was used to image blood volume under controlled conditions. This type of scan does not require injection of contrast or labeling agents. For each subject, 24 functional image data sets were acquired, in addition to anatomical images. Each trial consisted of one "rest state" scan (odorless air), followed by one "activated state" scan (odorant). Three types of odors were used eight times each: pleasant, unpleasant, and alcohol beverage odors. Each scan lasted 20 seconds, during which a three dimensional volume-image of 64 by 64 by 10 voxels was acquired. The pulse sequence, Echo-Shifted FLASH, renders an image which is sensitive to changes in the blood ratio of oxygenated versus deoxygenated hemoglobin, which in turn is thought to depend on local oxygen utilization.

Significant changes in signal intensity were found almost exclusively in brain structures involved in olfactory processing. Most foci of signal intensity change were located in secondary olfactory areas, such as amygdala, entorhinal cortex, nucleus accumbens/septal nuclei, and some in orbital frontal cortex. Different sites of changes were found in different subjects, possibly due to the relatively low sensitivity of this novel brain imaging method. Pleasant and unpleasant odors generally activated different olfactory structures, possibly reflecting the difference in affective evaluation.



Project Description:Investigators:

D. Hommer	Section Chief	LCS, NIAAA
S. Duyn	Staff Fellow	LDNR
C. Moonen	Director	NMR, NCRR
N. Ramsey	Visiting Fellow	LCS, NIAAA
R. Rawlings	Mathematical Statistician	LCS, NIAAA
D. Rio	Physicist	LCS, NIAAA
U. Ruttimann	Biomedical Engineer	LCS, NIAAA
P. Van Gelderen	Staff Fellow	NMR, NCRR

Objectives:

The purpose of this project is to develop the methods that allow us to measure changes in regional cerebral blood flow and/or blood volume that are associated with perception of odors. In essence, we are attempting to determine the functional neuroanatomy of olfactory perception in both normal controls and alcoholics.

Methods Employed:

A whole-body Signa 1.5 T scanner was used with a standard headcoil. Anatomical images were obtained using an inversion recovery sequence (TR 3000, TI 800, thickness 3 mm, gap 1 mm, 256 x 256, 45 degrees angle with AP plane). Functional scans were obtained from the same location, using a 3D Echo-Shifted Flash sequence (TE/TR = 30/20 ms), such that individual slices from the 3D volume overlapped exactly with the IR images. A data matrix of 64 x 64 x 16, total time 20 sec per 3D image resulted in a nominal resolution of 3.5 by 3.5 by 4 mm.

Pleasant odors (Muguet and Coconut), were administered to 10 subjects (ss), using a specially designed multivalve system. For stimulation, a clean continuous airstream (26°C, 50% humidity, 3.5 ltr.min<sup>-1</sup>) was directed through one of a choice of odorant chambers (2 oz. vials). A nasal cannula remained in place during the entire experiment. After anatomical scans, eight stimulation trials were done at three minute intervals. Each trial consisted of two consecutive functional scans, with a seven second interval between scans. Olfactory stimulation was switched on at the end of the first scan and off at the end of the second scan (timespan 27 seconds).

For each subject, the data were converted to difference images, obtained by subtracting the unstimulated from the stimulated 3D image within each trial. The intrasubject analysis method is based on properties of Gaussian random fields (adapted from Worsley et al.), and offers a statistical entity for significance of voxel (difference) values. These values are expressed as "X-values", i.e., the average difference per voxel, times the number of trials minus one, divided by the pooled standard deviation (PSD) (computed from all voxels in all the difference images). The volume of interest was limited to brain tissue. To minimize motion effects within and across trials, the original data sets were convoluted with a 2D gaussfilter (resulting full-width half-max 7.5 mm in x and y) prior to subtraction. Because the random field model assumes homogeneity of variances (over the difference images) across all voxels, the PSD was adjusted for the voxels with excessive variance (using the maximum of a Chi-square random field as a statistic). Signal intensity increase or decrease was regarded significant when the X-value of a voxel exceeded a threshold that was calculated for a probability of finding false significant voxels in one out of 10 subjects. The threshold corresponded to approximately 2.5% - 5% of the average signal intensity, depending on the subject (1.7 times the PSD, for eight trials).



Major Findings:

Functional images matched anatomical images very well (accuracy of one voxel or less at the perimeter of brain tissue). Therefore, no registration problems were encountered. Due to the susceptibility effects near the nasal cavity, signal along the olfactory nerve was lost (roughly 5 mm into brain tissue), including part of the primary olfactory cortex (piriform and anterior entorhinal cortex). However, most limbic structures, located posterior and superior to the cavity, were preserved. Significant voxels were found in structures of the olfactory system, including the amygdaloid complex (5 ss), septal nuclei (3 ss), ventromedial striatum (3 ss), posterior lateral orbito-frontal cortex (4 ss). Only a very small number of significant voxels were found outside of the olfactory system. We found no significant signal intensity changes in data sets (same size and number of trials) containing only rest-state difference images (no stimulation).

These preliminary results indicate that the 3D Echo-Shifted Flash sequence enables *in vivo* measurement of olfactory stimulation-induced signal intensity changes in human brain. The use of a random field model is a rigorous method for minimizing false positive results, while preserving acceptable statistical power. As a consequence, significant changes occurred almost exclusively in structures related to olfactory processing.

Significance to Biomedical Research and the Program of the Institute:

The physiological basis for desire to drink alcoholic beverages is poorly understood. Functional MRI of olfactory perception may provide a way of investigating this question.

Proposed Course:

Further development of the sequence and experimental procedures are necessary to understand: (a) the anatomical variation of significant voxels across subjects, (b) the absence of any significance in certain subjects (four out of 10 in this experiment), and (c) the effects of motion during and between trials on signal intensity changes. In addition, we plan to begin studies using alcoholic beverages as olfactory stimuli, and compare response of controls and alcoholic subjects. Finally, we plan to use food deprivation followed by food odors in normal controls to examine the effects of drive-state on olfactory response.

Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00064-03 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analysis of Brain Images

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. Rio Physicist LCS, NIAAA

Others: P. Andreason Senior Clinical Investigator LCS, NIAAA  
M. Eckardt Senior Investigator LCS, NIAAA  
D. Hommer Section Chief LCS, NIAAA  
R. Rawlings Mathematical Statistician LCS, NIAAA  
U. Ruttimann Biomedical Engineer LCS, NIAAA

COOPERATING UNITS (if any)

Experimental Therapeutics Branch, NIMH (J. Hsiao); MedData, McLean, VA (R. Momenan)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Electrophysiology and Brain Imaging

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

4.0

PROFESSIONAL:

2.5

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Traditional methods to analyze image data from PET and MRI have proven only partially successful. This is due in part to the inherent biological variability, physical limitations of the acquisition instrumentation, and mathematical algorithms applied to reconstruct the image data, but it also reflects the inadequacy of the computational, mathematical, and statistical methods employed in analysis of these data. Image data acquired by PET and MRI have numerous sources of distortion. Depending on the imaging modality, these appear as spatial distortions, decreases in signal-to-noise ratio, modification of image values, and increased spatial correlation. PET and MRI data can be "improved" by using appropriate models to restore and analyze the reconstructed image. In order to evaluate these models, simulated PET brain intensity data and PET and MRI brain shape data have been created using empirically measured image characteristics for PET and MRI. In particular, Monte Carlo techniques have been developed to create groups of PET data with known attributes and specific group differences. The control of signal and noise associated with these models allows us to evaluate the effect of geometric distortions and sensitivity of identification of localized statistically significant differences between the groups. In the case of geometric models it is possible to create 3-D brain (or skull) shapes with known noise to evaluate the limitations of rescaling of PET images across subjects to a given standard and registration of MRI and PET images for the same subject. These simulations are being used to study two related areas currently under investigation: (1) statistical techniques are being researched for both the geometric and grey scale values of PET and MRI data; and (2) the precision of multimodality 3-D superposition of functional and structural images obtained for PET and MRI data especially when the data are sparse or have known symmetries.

Project Description:Investigators:

D. Rio	Physicist	LCS, NIAAA
P. Andreason	Senior Clinical Investigator	LCS, NIAAA
M. Eckardt	Senior Investigator	LCS, NIAAA
D. Hommer	Section Chief	LCS, NIAAA
R. Momenan	Research Engineer	MedData, VA
R. Rawlings	Mathematical Statistician	LCS, NIAAA
U. Ruttimann	Biomedical Engineer	LCS, NIAAA

Objectives:

Traditional methods to analyze image data from PET and MRI have proven only partially successful. This is due in part to the inherent biological variability, physical limitations of the acquisition instrumentation, and mathematical algorithms applied to reconstruct the image data, but it also reflects the inadequacy of the computational, mathematical, and statistical methods employed in analysis of these data. Our goals are to reformulate, extend, or develop new techniques, which are mathematically and statistically well founded, that can be easily and clearly applied by a researcher or clinician to analyze these data.

Methods Employed:

Image data acquired by PET and MRI have numerous sources of distortion. Depending on the imaging modality, these appear as spatial distortions, decreases in signal-to-noise ratio, modification of image values, and increased spatial correlation. PET and MRI data can be "improved" by using appropriate models to restore and analyze the reconstructed image. In order to evaluate these models, simulated PET brain intensity data and PET and MRI brain shape data have been created using empirically measured image characteristics for PET and MRI. In particular, Monte Carlo techniques have been developed to create groups of PET data with known attributes and specific group differences. The control of signal and noise associated with these models allows us to evaluate the effect of geometric distortions and sensitivity of identification of localized statistically significant differences between the groups. In the case of geometric models it is possible to create 3-D brain (or skull) shapes with known noise to evaluate the limitations of rescaling of PET images across subjects to a given standard and registration of MRI and PET images for the same subject.

These simulations are being used to study two related areas currently under investigation: (1) Statistical techniques are being researched for both the geometric and grey scale values of PET and MRI data produced by the acquisition technique and reconstruction algorithms applied. These measures are then being used to both limit and extend current image analysis techniques. Special emphasis is being given to development of spatial measures in 2- and 3-D for the purpose of image averaging across subjects and the creation of different images between group averaged images. (2) The precision of multimodality 3-D superposition of functional and structural images obtained for PET and MRI data especially when the data are sparse or have known symmetries.

Major Findings:

Two simulated sets of 15 PET images were created consisting of constant elliptic regions with added Gaussian random noise (with zero mean). Each brain image within a group was given a constant offset, Gaussian distributed with zero mean for the group. Values for the simulated data were based on measurements from regional cerebral metabolic activity from actual PET data. No spatial transformations were applied to these images since they were created in exact spatial alignment.



Three statistical methods based on the Fourier transform, the wavelet transform, and the theory of Gaussian random fields in the spatial domain were applied to these data. Comparisons were then made among these techniques. Some initial results have suggested that all three techniques are relatively consistent as to their identification of localized regions associated with group differences. However, each statistical technique has its particular properties (see project on Statistical Analysis of Image Features). Specifically, this has led to modifications of existing Fourier image decomposition techniques and wavelet decomposition techniques. In the case of Fourier based statistical techniques applied within the frequency domain, specific image models which incorporate information about the scanner performance characteristics have provided a better measure of functional information and improved spatial resolution.

Brain image rescaling, consisting of multidimensional brain-shape standardization, has been implemented in order to enhance image information within and across groups of subjects. This enables the use of spatial averaging and signal extraction techniques in both the spatial and frequency domain to systematically analyze group differences. In this case, computer-generated brain models that stimulate brain image intensity and shape have indicated limitations with the rescaling algorithms currently being used. Thus, for a given signal-to-noise ratio associated with PET image intensities, biological variability due to brain shape may further confound the averaging techniques.

Spatial superposition/registration of PET functional data with MRI anatomic data is currently under investigation using established and enhanced methods to match PET and MRI data acquired from the same subject. In particular, these registration methods incorporate a number of techniques which include the following: (1) application of algorithms at multiple spatial resolutions, (2) multiple surface matching (including the intracerebral fissure), (3) direct "active" calculation of the transformation parameters from the data, and (4) use of a surface-weighted registration criterion. These techniques are being evaluated along with previously developed registration techniques to establish a benchmark for registration. Simulated data are being used to establish the limitations of these various methods. Additional verification of these methods will be accomplished by comparison with external fiducials placed on the subject during scanning and specifically designed to allow direct spatial registration. This will be used to study possible sources of error in 3-D graphical matching methods solely based on image data without fiducials.

Integration of many novel mathematical applications and image processing techniques has provided an elaborate set of tools to superimpose functional and anatomic images in 3-D as well as measure their effective registration error. These have been used to superimpose metabolic regions of interest derived from group averaged images onto MRI anatomic images. These techniques are currently being used by clinical researchers in a number of studies.

#### Significance to Biomedical Research and the Program of the Institute:

Clarification and verification of analysis technique as applied to the study of brain images of function and structure are critical. Establishing correct and reliable methods in this area of study will provide a cornerstone for further study within this field as well as allow integration of these data with electrical sources derived from scalp-monitored EEG and ERPs and psychometric data. Success combining these areas is a crucial step in determining brain mechanisms.

#### Proposed Course:

Images will be acquired during challenge conditions. MRI functional images will play a greater role within the context of our multimodality analysis technique. Simulations will provide information on experimental design including sample size

and sensitivity of statistical techniques to identify localized differences between functional images obtained from multiple groups or conditions. Simulations will also provide error estimates associated with anatomical localization associated with functional images by registration techniques.

Publications:

Rio DE, Rawlings RR, Ruttimann UE, Momenan R. A study of statistical methods applied in the spatial, wavelet and Fourier domain to enhance and analyze group characteristics of images: Application to PET brain images. In: Wilson DC, Wilson JN, eds. Proceedings Mathematical Methods in Medical Imaging II. Bellingham: The International Society for Optical Engineering, 1994, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00065-03 LCS

PERIOD COVERED

October 19, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Semi-Automated Methods of Segmentation of Brain Images

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: U. Ruttimann Biomedical Engineer LCS, NIAAA

Others: P. Andreason Senior Clinical Investigator LCS, NIAAA  
D. Hommer Section Chief LCS, NIAAA  
D. Rio Physicist LCS, NIAAA

COOPERATING UNITS (if any)

MedData, McLean, VA (R. Momenan)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Brain Electrophysiology and Imaging

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

1.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to achieve three-dimensional co-registration of images acquired with different technologies, corresponding landmarks must be identified in the respective images. The scale and orientation differences associated with image representations from different scanners present a major obstacle in this task. Visual identification of corresponding regions introduces subjectivity and inconsistency, and may become too laborious for large study series. Therefore, the aim of this research is the development of semi- or fully-automated computer methods for the identification and delineation ("segmentation") of important areas and landmarks (e.g., skull, gray and white brain matter, CSF, pathologic tissues) in brain images derived from different modalities. A semi-automated procedure that delineates in T1-weighted MR images the outer table of the skull and segments regions of CSF, gray and white brain matter, has been implemented. Its application to MR images of alcoholic and normal subjects yielded results consistent with a subjective segmentation. However, problems related to magnetic field inhomogeneities need to be overcome first to achieve reliable, unsupervised global segmentation, perhaps requiring the use of multivariate segmentation methods to incorporate both T1- and T2-image information.

For the purpose of enabling the registration of images that represent local glucose utilization (PET) with structural images (MRI or CT), algorithms for the automated detection of midlines in each slice were developed. Compared to midline placement by human operators, the detected midsagittal planes in PET images showed on average discrepancies of lateral displacement of 0.5 mm, and rotations in the transverse and coronal planes of 0.25 and 0.65 degrees, respectively. However, independent of the detection method, the midlines displayed systematic transverse rotations between subsequent slices, ranging from the highest to the lowest slice on average 2.5 degrees. Hence, the errors of automated midline detection were small compared to those incurred by assuming the midsagittal structures to lie in an ideal plane that neglects systematic contortions.

Project Description:Investigators:

U. Ruttimann	Biomedical Engineer	LCS, NIAAA
P. Andreason	Senior Clinical Investigator	LCS, NIAAA
D. Homner	Section Chief	LCS, NIAAA
R. Momenan	Research Engineer	MedData, McLean, VA
D. Rio	Physicist	LCS, NIAAA

Objectives:

An important step in the identification of neurologic mechanisms is the establishment of associations between structure and function among various areas of the brain. Since the acquisitions of either functional or anatomic images rely on different physical principles, different scanner technologies are involved which produce images at different spatial resolutions and orientations, as well as different temporal resolutions. These scale and orientation differences are major obstacles in the identification of corresponding regions in different image modalities. Hence, this research is directed to the implementation of semi- or fully-automated segmentation methods for images acquired with a variety of modalities.

Methods Employed:

Image segmentation is problematic because it implies "recognition", which is quantitatively poorly understood for tasks of real-world complexity. Consequently, "correct" measures of region homogeneity and thus, theoretically best methods to gauge performance in practical implementations, are lacking, leading to task-specific, heuristic approaches only loosely guided by general principles. However, for traditional reasons as well as mathematical tractability, mean-square metrics are most often used in developments and evaluations of segmentation performance. While manual outlining is considered the gold standard, it suffers several disadvantages because it depends on the operator's expertise, dexterity, and consistency, and tends to be slow, very tedious, and fatiguing. Most importantly, perceived boundaries depend on the display level settings of the monitor, giving rise to reproducibility problems.

In the registration of cross-modality images, the delineation of outer contours of the brain or skull is of particular interest. Skull contours are particularly useful for that purpose because of their mechanical rigidity and independence from brain function variations. Another useful landmark that can be reliably detected in both physiologic and anatomic images is the "midline" in each slice. In addition to the outlining of anatomic features, the certainty with which each point on that outline can be determined is of interest. This ancillary information can be used to derive appropriate weighting factors indicating the confidence in the location of each boundary point to be used in three-dimensional registration algorithms. Measures of location certainty under investigation are derived from local differential geometry and signal-to-noise ratio.

An issue related to the precision of anatomic localization is the effect of head movement during scan acquisition, and in particular between repetitive scans obtained under different states of stimulation within a subject. Head movement results in loss of registration accuracy, resulting in misregistration artifacts and thus, loss of sensitivity to detect between-scan differences of biological origin. Hence, it is the interest to quantify head movements and assess their impact on scan registration.

Major Findings:

(1) Segmentation methods previously developed for CT images have been extended toward application of MRI images. The challenge with this modality is that image contrast of the different tissues changes or even reverses with different weightings of the T1 and T2 signal components. Another problem is that the skull, a stable landmark for image registration across modalities, is only indirectly characterized by the absence of an MR signal. Automated recognition of the scalp signal in T1 images with delineation of the outer table of the skull has been implemented by determination of appropriate thresholds from intensity histograms and found satisfactory in a preliminary evaluation. Also, separation of gray from white brain matter, and from CSF based on these methods, could be achieved in T1 images. However, problems related to magnetic field inhomogeneities need to be overcome first to achieve reliable, unsupervised global segmentation. Furthermore, the signal contrast between CSF and bone has been found insufficient in T1 images to attain reliable detection of the inner table of the skull, suggesting the development of segmentation methods for vector quantities so that T1 and T2 image information can be combined.

(2) Segmentation methods for PET images representing local glucose utilization have been developed to enable three-dimensional co-registration with CT or MRI scans. Methods to detect the scalp signal indicating the outlines of the skull, which is a function-independent anatomic landmark, have been developed previously. The midline in the slices, which is to a large extent also independent of function, was selected as a second registration landmark. Algorithms for detection of this feature have been developed that are based on three different techniques of assessing line-by-line differences between the left and right sides of the brain: (a) phase differences of the Fourier transforms, (b) mean absolute differences of axial-symmetric pixels, and (c) maximum number of zero crossings in the left-right difference signal. A comparison of the methods to midline placement by human observers on 630 slices indicated method (a) to perform best with mean discrepancies of the lateral displacement of the detected midsagittal planes of about 0.5 mm, and rotations in the transverse and coronal planes of 0.25° and 0.65°, respectively. However, the midlines displayed systematic transverse rotations between subsequent slices, ranging from the most superior to the most inferior slice, on average 2.5°. Hence, the errors of automated midline detection were small compared to those incurred by assuming the midsagittal structures to lie in an ideal plane that neglects systematic contortions.

Significance to Biomedical Research and the Program of the Institute:

This project is part of the general research aimed at the understanding of associations of brain function with structure and their possible alterations in alcoholic individuals. This requires the three-dimensional spatial superposition of information acquired by different imaging modalities, for which segmentation is of central importance. Its automation will increase the objectivity of the mensuration processes, enable feasibility of large scale-studies, and improve sensitivity for detection of image differences between subject groups or between functional states in challenge paradigms.

Proposed Course:

Segmentation problems associated with magnetic field inhomogeneities in MR images will be investigated and methods for their amelioration developed. Methods for the segmentation of vector quantities will be developed to investigate their potential for improved segmentation of co-registered T1, T2 and, possibly, proton density images. The midline detection method developed for PET images will be applied and evaluated for MR images.

Publications:

Ruttimann UE, Andreason PJ, Rio D. Head motion during PET scanning: Is it significant?, Psychiatry Research: Neuroimaging, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

201 AA 00082-01 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Statistical Analysis of Image Features**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: U. Ruttimann Biomedical Engineer LCS, NIAAA

Others: P. Andreason Senior Clinical Investigator LCS, NIAAA  
M. Eckardt Senior Investigator LCS, NIAAA  
D. Hommer Section Chief LCS, NIAAA  
R. Rawlings Mathematical Statistician LCS, NIAAA  
D. Rio Physicist LCS, NIAAA

COOPERATING UNITS (if any)

MedData, McLean, VA (R. Momenan), BEIP, NCRR (M. Unser)

LAB/BRANCH

**Laboratory of Clinical Studies**

SECTION

**Section of Electrophysiology and Brain Imaging**

INSTITUTE AND LOCATION

**NIAAA, 9000 Rockville Pike, Bethesda, MD 20892**

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The final step in medical imaging is often the assessment of the statistical significance of local differences between average images of different subject groups, or of the same subjects at different functional states. The aim of this project is the development of statistical methods that either take into account the spatial correlation of images, or apply image transform methods that permit an unbiased decomposition of images into uncorrelated components. Three statistical methods are being developed based on the Fourier transform, the wavelet transform, and the theory of Gaussian random fields. In the Fourier domain, the statistics at different wave numbers are uncorrelated and statistical tests can be performed unencumbered by spatial correlations. This method suffers from poor spatial localization but provides for rigorous statistical testing. However, by taking into account image blurring constraints, localization may be significantly improved. Statistical tests in the Fourier domain were performed on PET images obtained from alcoholic and normal subjects, which identified approximately 1,500 (of 8,000) wave numbers that were significantly different between the groups. The wavelet transform is a recently discovered decomposition method that permits a tradeoff between the sharpness of localization in the image domain and the decorrelation of image components. This method decomposes the image into sets of wavelet coefficients in nearly uncorrelated channels of successively decreasing resolutions, permitting separate statistical testing of the coefficients at each resolution level. Application of this method to PET images indicated only 260 (1.6%) of the coefficients to be statistically different from noise, which then served to resynthesize the relevant differences in the spatial domain. Applying the theory of Gaussian random fields on the same PET data identified similar regional differences at an overall p-value of .05. Correlation of image components with external variables (e.g., age) was also possible using this theory.



Project Description:Investigators:

U. Ruttimann	Biomedical Engineer	LCS, NIAAA
P. Andreason	Senior Clinical Investigator	LCS, NIAAA
M. Eckardt	Senior Investigator	LCS, NIAAA
D. Hommer	Section Chief	LCS, NIAAA
R. Momenan	Research Engineer	MedData, VA
R. Rawlings	Mathematical Statistician	LCS, NIAAA
D. Rio	Physicist	LCS, NIAAA
M. Unser	Visiting Scientist	BEIP, NCRP

Objectives:

The final step in medical imaging is often the assessment of the statistical significance of certain image features of interest, such as local differences between average images of different subject groups, or of the same subjects at different functional states. In many studies, the statistical models are ill-applied, the underlying hypotheses not clearly stated or inconsistent with the models used. Therefore, the aim of this project is the development of statistical methods that either take into account the correlation of image intensities among neighboring pixels, or apply global image transform methods that permit an unbiased decomposition of images into uncorrelated components. Of particular importance for such transforms is that they maintain "good localization" in the original image domain, so that the results of statistical analyses can be referred back to the original image for display of the "significant" features. Of further interest is to develop methods enabling the assessment of the correlation of image components with external variables, such as age or neuropsychological measures.

Methods Employed:

Currently, three statistical methods to analyze and compare images acquired from one or more groups of subjects are being developed based on the Fourier transform, the wavelet transform, and the theory of Gaussian random fields in the spatial domain.

In the Fourier domain, the statistics at different wave numbers are essentially uncorrelated and statistical tests can be performed unencumbered by the existing spatial correlations. In general, this method suffers from poor spatial localization but provides for rigorous statistical testing. However, by incorporation of appropriate blurring approximations, localization may be significantly improved. This may be of particular value in producing unbiased estimates of the entire difference image between two groups of subjects or in classification where spatial localization is not a problem.

The wavelet transform is a recently discovered decomposition method that bears some relationship to the Fourier transform, but has the theoretical advantage of achieving a flexible tradeoff between the sharpness of localization in the image domain and the decorrelation of image components. This method decomposes the image into a set of approximation images with successively decreasing resolution levels, then transforms the differences between adjacent resolution levels into an orthogonal space of functions ("wavelets"), the amplitudes of which ("wavelet coefficients") suffice to fully reconstruct the original image. The wavelet coefficients at different resolutions are nearly uncorrelated, permitting statistical testing of the coefficients at each resolution level separately.

Finally, building on work recently developed for the analysis of images in the spatial domain using the theory of Gaussian random fields, it is possible to construct images to test various statistical hypothesis. These include a z-

map image to test for significant local differences between groups of images and a t-map image to test for localized correlation of a group of images with an external variable. With both these fields it is possible to establish a threshold for statistical significance based on the resolution of the acquired images.

#### Major Findings:

Statistical tests in the Fourier domain were performed on two groups of PET images obtained from alcoholic and normal subjects, which identified at a p-value of .10 approximately 1,500 wave numbers (out of 8,000) that were significantly different between the groups. Using only these wave numbers to reconstruct the image produced images that were similar but more diffuse than the original difference images. This resulted in a decrease in the mean square error for the difference image, and the wave numbers which were found significant corresponded in general to a low-pass filter of 7 mm. Linear discriminant analysis applied in the Fourier domain for a sample of three slices between the groups resulted in 100% correct classification (using resubstitution) for the one slice which showed a localized difference and 75% correct classification with the leaving-one-out methodology.

An orthogonal wavelet decomposition was applied to images representing group-differences in PET images obtained from alcoholic and normal subjects. This produced wavelet coefficients in 15 channels at five resolution levels, which could then be analyzed for statistical significance against independent image noise by F-tests. Application of the corresponding 15 tests at an overall (Bonferroni adjusted)  $p=0.05$  resulted in the global rejection of all wavelet coefficients at the two highest resolution levels. This eliminated 94.8% of the total number of coefficients, requiring only the remaining coefficients to be tested individually. These follow-up tests identified 260 (1.6%) of the coefficients as statistically significant, which then served to resynthesize by inverse wavelet transform the relevant differences in the original image domain. Due to the excellent localization property of the selected wavelet family, these images displayed uniform, relatively artifact- and noise-free regions of local glucose utilization differences.

Applying the theory of Gaussian random fields on the data sets previously mentioned identified similar regional differences as seen in the images enhanced by Fourier and wavelet statistical methodology. Furthermore, using this theory it was possible to identify significant regional differences at an overall p-value of .05. Lastly, regional correlation for a group of alcoholics images with the external variable of age (highly indicative of lifetime alcoholic consumption) was produced.

#### Significance to Biomedical Research and the Program of the Institute:

Uncritically used statistical analyses may be forgiving when applied to images displaying relatively large differences in areas where such differences were expected to occur by some a priori reasoning. While they may only provide overly optimistic p-values in such situations, their use is scientifically unsound in the case of small differences that may appear in "unexpected" areas, which, if true, may constitute truly new findings. Hence, strengthening of the statistical inference tools for image applications will increase the objectivity of the conclusions in a variety of imaging studies.

#### Proposed Course:

In the Fourier domain, incorporation of the blurring function as suggested by its use with Gaussian random fields will be further investigated. Initial indications are that it will significantly improve spatial localization. Isotropy of the selected wavelet family will be investigated, and if found insufficient, two-dimensional wavelet transforms defined on a hexagonal grid

will be explored. Gaussian random field methods will be developed to establish parametric testing procedures for individual wavelet coefficients. The Gaussian random field theory will be extended to cover experimental setups which include multiple groups with multiple conditions, unequal group sizes, and correlation with external variables. The sensitivity and statistical power of these methods will be investigated in simulated images.

Publications:

Ruttimann UE, Unser M, Rio DE, Rawlings RR. Use of the wavelet transform to investigate differences in brain PET images between patient groups. In: Wilson DC, Wilson JN, eds. Proceedings Mathematical Methods in Medical Imaging II. Bellingham: The International Society for Optical Engineering, Vol. 2035, 1993; 192-203.

Rio DE, Rawlings RR, Ruttimann UE, Momenan R. A study of statistical methods applied in the spatial, wavelet and Fourier domain to enhance and analyze group characteristics of images: Application to PET brain images. In: Wilson DC, Wilson JN, eds. Proceedings Mathematical Methods in Medical Imaging II. Bellingham: The International Society for Optical Engineering, 1994, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 AA 00063-03 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

EEG Studies of Electromotive Generators Affected by Alcohol

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. Westdorp	IRTA Fellow	LCS, NIAAA
Others:	C. Adams	IRTA Fellow	LCS, NIAAA
	M. Eckardt	Senior Investigator	LCS, NIAAA
	S. Law	Special Volunteer	LCS, NIAAA

COOPERATING UNITS (if any)

Dept. Psych., Wash. U (J. Rohrbaugh); Brain Phys. Group, Tulane U (P. Nunez); Dept. Computer Sci., Rensselaer Polytech. Inst. (J. Goble); INSERM CJF 90-12 (Rennes, France)/Brain Inst., UCLA (E. Halgren); Biomed. Eng. & Instr. Br., NIH (B. Roth)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Brain Electrophysiology and Imaging

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects   ☐ (b) Human tissues   ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The long-term objective of this research is the quantification of acute and chronic effects of alcohol on electromotive generators in the brain, and the determination of relationships of abnormal generators and predisposition to excessive alcohol consumption.

Research integrates the development of high-resolution EEG methods to localize and study electromotive generators of brain processes with various electrophysiological models, both computational and theoretical, to validate the methods and test hypotheses of suspected generators. This integrated procedure is used to determine how, when, and where alcohol alters mechanisms which process sensory input, respond to input, and store and retrieve information. Event-related brain electrical potentials (ERPs), which provide both spatial and temporal information about these mechanisms, and nonstationary EEGs, which provide a measure of the brain's dynamic interactions, are used in this investigation. A physical, artificial head model is also used to study the inhomogeneous and anisotropic nature of volume conduction in the head.

Methods have been developed that improve the spatial resolution of current EEG methods by a factor of three. Preliminary findings suggest that temporal resolution is improved as well. These methods have been shown to provide better estimates of underlying brain processes than present techniques and are more insensitive to electrophysiological artifacts, such as eye blinks. These new techniques are currently being used to analyze EEG data of individuals with different family histories of alcoholism.



Project Description:Investigators:

A. Westdorp	IRTA Fellow	LCS, NIAAA
C. Adams	IRTA Fellow	LCS, NIAAA
M. Eckardt	Senior Investigator	LCS, NIAAA
J. Goble	Visiting Assistant Professor	Rensselaer Polytech. Inst., Troy, NY
E. Halgren	Professor	INSERM CFS 90-12 Rennes, France/UCLA
S. Law	Special Volunteer	LCS, NIAAA
P. Nunez	Professor and Director	Brain Physics Group, Tulane University, New Orleans, LA
J. Rohrbaugh	Associate Professor	Washington U, St. Louis, MO
B. Roth	Biomedical Engineer	Biomedical Eng. & Instrumentation Branch, NIH

Objectives:

This research is designed to: (1) develop accurate spatial and temporal resolution EEG methods to localize and characterize electromotive generators of underlying brain processes. EEG spatial resolutions on the order of magnitude of MRI data (1-2 mm) that can be assessed with data derived from other imaging modalities (PET, MRI, MEG, MRS) are sought; and (2) develop electrophysiological models which can be used to study how the brain processes information.

Methods Employed:

Theoretical high-resolution EEG models and methods are developed using known electrophysiologic transmission characteristics of the human head that best account for its inhomogeneous and anisotropic nature. Current-density methods are employed because, unlike potential-based methods, they are severely affected by volume conduction distortion. Current-density is not dependent on volume conduction effects and enhances local activity while minimizing global contributions at a given electrode.

Three methods are employed to study the variational aspects of volume conduction in the human head. First, conductance studies of human bone are used to estimate the conductances over various regions of the human skull. Second, physical human skull models, constructed with materials of known electrical conductivity, are used to study the geometric properties of current flow in the human head (in collaboration with P. Nunez and J. Goble). Previous studies used dried human bone that may not reflect physiologic values. Third, scalp and intracranial EEG data, from electrodes which are applied during surgical treatment of intractable epilepsy (in collaboration with E. Halgren), are used to obtain local tissue resistances. The results of these studies provide parameters that account for local variations in current flow.

Computational models are used to study how signals from electromotive generators are transmitted from the brain, through the skull, to the scalp. Analytic models, such as the 3-sphere model of the head, are used to provide a first approximation. Complex models based on realistic head shape and tissue components are then applied to obtain a more accurate model. Data from MRI and CT are used to construct these models. Three volume-conduction models are to be used: a Finite Element Model (FEM) developed by the Tulane Brain Physics Group (P. Nunez), a Boundary Value Model developed by B. Roth, and a Finite Difference Model (FDM) developed by the Rennes group (E. Halgren) in collaboration with this

Section. All models have been validated analytically and are currently in the process of incorporating data from MRI and CT.

Brain electrical activity and other psychophysiological responses are measured from up to 48 surface electrodes using conventional EEG instruments. Both ERP and nonstationary EEG are recorded and utilized. The ERPs are analyzed with respect to wave form and sensitivity to experimental variables using multivariate techniques. Nonstationary EEG is analyzed with respect to coherence and other measures of dynamic processes. Topographic distributions of the responses over the scalp are studied for determination and identification of neural sources of the electrical activity using scalp mapping and dipole inference techniques. These results are then compared with the computational models to estimate and test likely locations of electromotive generators.

In summary, development and improvement of high-resolution EEG methods is accomplished through application of algorithms which take into account the electrophysical nature of the human head and the incorporation of parameters which account for local variations. The findings from these studies are used to construct and improve electrophysiologic models of brain processes. The results from both simulated and human data are used in hypothesis testing to localize and characterize how, when, and where alcohol alters mechanisms associated with information processing and cognition in the brain.

#### Major Findings:

Dr. Law left NIH in June of 1993. A new Fellow, Andrew Wesdorp, has been recruited to take his place. However, he did not arrive until late in the year and as a result there are no new major findings to report for this project.

#### Significance to Biomedical Research and the Program of the Institute:

The ability to determine accurately electromotive generators in the brain is of great significance because it will allow investigators to localize dynamic processing of information. Once this is accomplished, the acute and chronic effects of alcohol consumption on the processes can be studied.

#### Proposed Course:

Data will continue to be collected.

#### Publications:

Law SK, Nunez PL. High resolution EEG using spline generated surface Laplacians on spherical and ellipsoidal surfaces, IEEE Trans Biomed Eng, in press.

Law SK, Rohrbaugh JW, Adams CM, Eckardt MH. Improving spatial and temporal resolution in stationary EEG using spline generated surface Laplacians, Electroencephalogr Clin Neurophysiol, in press.

Law SK. Thickness and resistivity variations over the upper surface of the human skull, Brain Topogr, in press.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00004-01 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hepatitis C Virus Infection in Alcoholics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. George Senior Clinical Investigator LCS, NIAAA

Others: M. Eckardt Senior Investigator LCS, NIAAA

D. Herion Senior Staff Fellow LCS, NIAAA

COOPERATING UNITS (if any)

LDS, NIDDK (H. Conjeevaran)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Studies

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOXES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

In this protocol we will evaluate the patterns and severity of liver disease in alcoholics who are infected with hepatitis C virus (HCV). Comparison groups include nonalcoholics (abstainers and nonabusive consumers of alcohol) infected with HCV and alcoholics who are not infected with HCV. Measures of liver disease include the histologic activity index (Knodel Scoring) for chronic active hepatitis, as well as noninvasive and functional studies, including transaminase fluctuations over time and changes in blood bile salt levels in response to a nutritional stimulus. Additional studies include measurement of urinary 24 hour cortisol excretion and CSF levels of CRH and ACTH to determine whether hypothalamic-pituitary-adrenal axis activation in alcoholics has a negative impact on liver disease. The response of lymphocytes (obtained from the peripheral blood and liver) to both nonspecific and specific (i.e., HCV protein and/or peptide) antigens will also be studied during alcohol withdrawal and at various times of abstinence from alcohol.

Preliminary results show that liver biopsy specimens obtained from asymptomatic alcoholics with HCV have revealed various degrees of chronic active hepatitis including the development of fibrosis and cirrhosis. Other studies, based on limited numbers, suggest a diminished peripheral blood mononuclear cell responsiveness to nonspecific antigens in the HCV-infected, viremic alcoholics.

Project Description:Investigators:

D. George	Senior Clinical Investigator	LCS, NIAAA
H. Conjeevaran	Senior Staff Fellow	LDS, NIDDK
M. Eckardt	Senior Investigator	LCS, NIAAA
D. Herion	Senior Staff Fellow	LCS, NIAAA

Objectives:

In this project we are attempting to characterize HCV infection in alcoholics. We wish to determine whether and how HCV may be modulated by alcoholism. Specifically, we will compare the severity of liver disease, the host immune response, and virologic factors (such virus genotype and levels of virus in the blood) during periods of alcohol consumption and periods of prolonged abstinence.

Methods Employed:

Alcoholics who have not used IV drugs in the last six months are eligible to participate. Blood for immunologic and virologic studies and urine for cortisol determinations are collected serially during the hospital stay. Lumbar punctures are performed within a week after admission. Liver biopsies are performed during the third or fourth week of abstinence from alcohol. The bile salt study is performed on the day of the liver biopsy.

Major Findings:

Liver biopsies performed on HCV positive alcoholics (clinically asymptomatic for liver disease) have shown the presence of various stages of chronic active hepatitis and, in some cases, cirrhosis. We have noted exacerbations of transaminase elevations in some HCV positive alcoholics during withdrawal, particularly in two individuals who have cirrhosis. This is in contrast to other studies which have shown serologic improvement (indeed clearing) of HCV infection after alcohol withdrawal. One noninfected alcoholic has undergone biopsy which showed chronic active hepatitis, a lesion which is thought to be very rare in alcoholics.

Significance to Biomedical Research and the Program of the Institute:

This study will help clarify the role of alcoholism in the pathogenesis of HCV infection. It may also provide interesting data on how various aspects of the immune response may be altered in chronic HCV infection and establish whether this response changes during periods of fluctuations in liver disease activity. Finally, we hope to generate data supporting possible bi-directional relationships between the neuroendocrine axis and immune response. The finding of exacerbations of liver disease during periods of alcohol abstinence would have important clinical implications for the treatment and monitoring of abstinent, HCV positive alcoholics.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00066-03 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Psychological & Biological Study of People Who Exhibit Abusive Behavior Patterns

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. George Senior Clinical Investigator LCS, NIAAA

Others: G. Brown Clinical Director LCS, NIAAA  
M. Eckardt Senior Investigator LCS, NIAAA  
M. Linnoila Scientific Director NIAAA  
P. Ragan Senior Clinical Associate LCS, NIAAA  
J. Umhau Clinical Investigator LCS, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Science

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.5

PROFESSIONAL:

0.4

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Domestic violence involving both children and adults is a problem of growing national concern. Both our clinical experience and a published report indicate that a significant number of subjects who perpetrate these abusive acts may have an underlying diagnosis of panic disorder. We postulate that the mental state of "being out of control", frequently described by these individuals during the abusive act, is linked to the pathophysiology of panic disorder. To test this hypothesis and to elucidate unique psychological and biological characteristics of patients who perpetrate abusive acts, we will compare subjects who have panic symptoms (e.g., "loss of control") and become aggressive with: (1) subjects who are aggressive but don't have panic symptoms; and (2) normal volunteers. These comparisons will consist of psychosocial and family histories, pharmacological challenge studies, and a determination of cerebral spinal fluid metabolites, as well as a careful analysis of the precipitating events associated with the violence. Preliminary results show that individuals who initiate acts of domestic violence have a higher than expected prevalence of alcoholism/abuse, panic disorder, and obsessive compulsive personality disorders. Clinically, patients described a number of physical symptoms as well as cognitive symptoms prior to initiating the violence.

Project Description:Investigators:

D. George	Senior Clinical Investigator	LCS, NIAAA
G. Brown	Clinical Director	LCS, NIAAA
M. Eckardt	Senior Investigator	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
P. Ragan	Senior Clinical Associate	LCS, NIAAA
J. Umhau	Clinical Investigator	LCS, NIAAA

Objectives:

In the United States there is an increasing awareness and concern about domestic violence. It is estimated that at least three million women each year are victims of violence perpetrated by their male companions. This estimate is probably low since most violent incidents occur in the privacy of the home and many are not reported to authorities. Statistics involving female perpetrators are even less well delineated. Also of great concern is the increasing number of reports of physical and sexual abuse of children. For example, one study noted that there were 6,000 reports of children being abused in 1976 and 100,000 in 1984.

There is an historically recognized relationship between alcohol abuse and violence. For example, 59% of offenders convicted of violent crimes used alcohol just before the offense. According to a study by Schuerger, more than 60% of men who battered women were alcohol abusers. When alcohol abuse and physical abuse occur concomitantly, alcohol consumption is thought to disinhibit social restraints and facilitate aggressive acts. While this may be true in some situations, it is possible that for some individuals alcohol serves to self-medicate affective states which in themselves may give rise to abusive behavior. For example, we have studied several alcoholics with panic disorder who give accounts of using alcohol to feel "in control" and dampen feelings of anxiety. Many of these individuals described aggressive behavior toward family members during periods of both sobriety and alcohol ingestion. Treatment with antidepressants resulted in a decrease in both fear and aggressive symptoms.

Methods Employed:

Both men and women who have a history of inflicting physical abuse are eligible to participate in the protocol. Prior to participation in the study, all subjects will be carefully evaluated to assure they are in good physical health and conform to predetermined diagnostic criteria. Specific components of the protocol include: complete psychiatric evaluation including a SCID I and II; a battery of psychological tests; lactate infusions; lying/standing NE determination; and lumbar puncture.

Major Findings:

Twenty-two subjects have been enrolled in the protocol to date. These subjects have a higher prevalence of alcoholism/abuse, panic disorder, borderline personality, and obsessive compulsive personality. Clinically, patients described a number of physical symptoms (palpitations, shortness of breath, shaking, sweating) as well as cognitive symptoms (losing control, depersonalization, fear) prior to "going off" and becoming violent. Following lactate administration, approximately 50% of the patients reported heightened states of arousal accompanied by feelings of fear and/or aggression. Results for the lying/standing norepinephrine challenge study as well as the lumbar puncture await analysis. Eight PET scans have been performed. Preliminary results (n=2) from the PET scan data show a decrease in glucose utilization in the orbitofrontal brain region.



Significance to Biochemical Research and the Program of the Institute:

If it is possible to link panic disorder through biological mechanisms or psychosocial histories to violent behavior, then it is probable that pharmacological interventions (such as antidepressants which have been useful in treating panic disorder) may also be efficacious in preventing or attenuating violent acts among this subgroup. Used as an adjunct to traditional behavioral interventions, the combination of pharmacotherapy and behavioral intervention may prove to be synergistic in preventing future family violence. It is also hoped that the information generated by this research will provide a basis for development of prevention strategies for this important subgroup of perpetrators of violence.

Publications:

Bitler DA, Linnoila M, George DT. Psychosocial and diagnostic characteristics of individuals initiating domestic violence, J Nerv Ment Dis, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 00067-03 LCS
PERIOD COVERED <b>October 1, 1993 to September 30, 1994</b>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Psychological and Biological Characterization of Smoking Withdrawal in Alcoholics</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <b>PI: D. George Senior Clinical Investigator LCS, NIAAA</b>  <b>Others: M. Eckardt Senior Investigator LCS, NIAAA</b> <b>P. Ragan Senior Clinical Associate LCS, NIAAA</b> <b>R. Rawlings Mathematical Statistician LCS, NIAAA</b>		
COOPERATING UNITS (if any) <b>Medical Psychology, USUHS (N. Grundberg)</b>		
LAB/BRANCH <b>Laboratory of Clinical Studies</b>		
SECTION <b>Section of Clinical Studies</b>		
INSTITUTE AND LOCATION <b>NIAAA, 9000 Rockville Pike, Bethesda, MD 20892</b>		
TOTAL STAFF YEARS: <div style="text-align: center;"><b>1.5</b></div>	PROFESSIONAL: <div style="text-align: center;"><b>1.0</b></div>	OTHER: <div style="text-align: center;"><b>0.5</b></div>
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>             Alcoholics are more likely to smoke cigarettes than individuals without a drinking problem. The nature of the interaction between smoking and drinking alcohol is poorly understood and raises important questions about: (1) advisability of giving up two addictions at one time, and (2) effects that continued smoking may have on the rate of relapse in patients with alcoholism. In this protocol, we are studying the psychological, pharmacological, and physiological effects of smoking cessation in patients with alcoholism. This will lead to a better understanding of chemical dependency in general, and to more effective treatment methods for both nicotine and alcohol dependency. Preliminary results demonstrate that alcoholics are able to successfully abstain from both alcohol and nicotine simultaneously in a controlled environment. Specific psychological and biological measures that characterize withdrawal await statistical analysis.           </p>		

Project Description:Investigators:

D. George	Senior Clinical Investigator	LCS, NIAAA
M. Eckardt	Senior Investigator	LCS, NIAAA
N. Grundberg	Professor	Medical Psychology, USUHS
P. Ragan	Senior Clinical Associate	LCS, NIAAA
R. Rawlings	Mathematical Statistician	LCS, NIAAA

Objectives:

Alcoholism and nicotine dependence have an extremely high rate of comorbidity. Studies indicate that 70% to 97% of alcoholic patients smoke cigarettes. This link between nicotine dependence and alcoholism is reinforced by studies showing a positive correlation between alcohol ingestion and increased smoking. Alcohol abuse is strongly associated with an increased number of cigarettes smoked per day, higher potency of cigarettes, and an increase in the inhalation volumes. Yet, in spite of these findings, cigarette smoking is rarely addressed in the context of alcohol treatment programs. This arises from the following widely held beliefs: (1) alcoholism is a more severe problem than nicotine dependence; (2) it is too difficult to give up two addictions simultaneously; and (3) cessation of both may increase the risk of relapse to drinking.

The need to address the issue of smoking withdrawal in the context of alcohol treatment is highlighted by studies showing that the successful cessation of one drug may be related to the successful cessation of another drug. For example, alcoholics who successfully quit smoking show a higher rate of abstinence than alcoholics who continue to smoke. Furthermore, relapse to smoking may be related to a relapse to drinking. Thus, treatment programs that address both addictions may yield synergistic effects.

In this protocol, we are primarily concerned with characterizing the effects of smoking cessation in alcoholics recently detoxified from alcohol. Specifically, we are asking the question: Do alcoholics who abstain from alcohol and nicotine simultaneously experience significantly more withdrawal symptoms than alcoholics who continue to smoke? To answer this question, we propose to study a number of alcoholic patient groups and compare psychological variables, autonomic reactivity, changes in central nervous system metabolism, caloric consumption, and immunological competency.

Methods Employed:

Prior to participation in this study, all subjects are carefully evaluated to assure they are in good health and conform to predetermined diagnostic criteria. Alcoholic subjects are then acclimatized to the unit for approximately one week prior to the start of biological studies. After being advised of the protocol, alcoholic subjects who smoke are asked to participate in a randomizing process which assigns them to either a "smoking cessation" or "continue smoking group." Alcoholic subjects who don't smoke are also asked to participate in the protocol. Smokers not willing to enter the randomizing process are not eligible for participation.

All subjects participate in a number of procedures (e.g., calorie counts, lumbar puncture, lying/standing norepinephrine determination, and idazoxan infusions) aimed at understanding how smoking cessation and alcoholism ultimately effect caloric consumption, central nervous system neurotransmitters, and autonomic nervous system reactivity.

Major Findings:

To date, 31 alcoholics have been randomized to either stop smoking (17) or to continue smoking (14). Of those randomized to stop smoking, five relapsed to smoking within the first week of the study; an additional three relapsed at the conclusion of the study. Preliminary data analysis shows: (1) At baseline, alcoholics consumed more calories over a 36 hour period than controls. This resulted from increased fat and protein consumption. Alcoholics who stopped smoking had increased calorie consumption compared to those who continued to smoke. (2) Physiological data acquired during and after the idazoxan infusion showed no difference in heart rate between alcoholics who stopped or continued to smoke. This is in contrast to systolic BP (AUC) which was greater in alcoholics who stopped smoking. No new patients have been added to the study since the last review pending the analysis of the following: (a) neuroendocrine responses to the double-blind idazoxan infusion; (b) idazoxan blood levels during the infusion; (c) serial nicotine/cotinine measurements (serum) of each subject throughout the study; (d) neutral amino acid measurements following the liquid test meal; and (e) CSF catecholamine metabolite determinations.

Significance to Biomedical Research and the Program of the Institute:

It is hoped that by studying the psychological and biochemical effects of smoking cessation in alcoholics, we can gain a better understand of chemical dependency which, in turn, will lead to more effective treatment methods for both nicotine and alcohol dependency.

Publications:

None.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  201 AA 00274-06 LCS
PERIOD COVERED <b>October 1, 1993 to September 30, 1994</b>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Intravenous Procaine in Alcoholics and Adult Children of Alcoholics</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <b>PI: D. George Senior Clinical Investigator LCS, NIAAA</b>  <b>Others: M. Linnoila Scientific Director NIAAA</b>		
COOPERATING UNITS (if any) <b>Biological Psychiatry Branch, NIMH (R. Post); Clinical Brain Disorders Branch, NIMH (R. Coppola)</b>		
LAB/BRANCH <b>Laboratory of Clinical Studies</b>		
SECTION <b>Section of Clinical Studies</b>		
INSTITUTE AND LOCATION <b>NIAAA, 9000 Rockville Pike, Bethesda, MD 20892</b>		
TOTAL STAFF YEARS: <div style="text-align: center;">3.0</div>	PROFESSIONAL: <div style="text-align: center;">2.5</div>	OTHER: <div style="text-align: center;">0.5</div>
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Previous studies show that the administration of procaine to bipolar and borderline patients gives rise to a diversity of behavioral responses ranging from euphoria to dysphoria. Since procaine is believed to selectively stimulate the limbic structures of the brain, it is reasoned that these behavioral differences might result from neuroclinical differences in these regions of the brain. To test the hypothesis that alcohol might alter limbic function, we administered procaine to alcoholics, patients with panic disorder, panic patients with alcoholism, and controls. Our results show that patients with panic disorder were more likely to experience a panic attack following procaine than were controls. Although the groups differed significantly in their behavioral response to procaine, there were no significant differences between patients and controls for procaine induced changes in norepinephrine, epinephrine, cortisol, ACTH, or prolactin.</p>		

Project Description:Investigators:

D. George	Senior Clinical Investigator	LCS, NIAAA
R. Coppola	Engineer	CBDB, NIMH
M. Linnoila	Scientific Director	NIAAA
R. Post	Chief	BPB, NIMH

Objectives:

The present study will address the hypothesis that abstinent alcoholics who have experienced a number of withdrawal bouts might have abnormalities of limbic function that will be revealed by procaine infusion. In addition, it is possible that temporal lobe dysfunction and kindling processes may occur in patients with panic disorder. In our experience, a large number of alcoholics have had panic attacks. We shall, therefore, study a group of alcoholics who have panic disorder as well as alcoholism. To eliminate the possibility that any changes found might reflect predisposition to alcoholism, a sample of adult nonalcoholic offspring of alcoholics will also be recruited and tested. These will comprise a group that have had panic attacks and a group that is free of them.

Methods Employed:

Prior to participation in the study, all subjects will be carefully evaluated to assure they are in good physical health and conform to predetermined diagnostic criteria. During the study, subjects receive placebo followed by two intravenous doses of procaine hydrochloride. Individuals are then carefully monitored for drug-induced changes in physical measures (pulse, blood pressure, vagal tone), mood, hormones (prolactin, ACTH, cortisol), and EEG activity.

Major Findings:

To date, 14 alcoholics, 13 controls, 11 alcoholics with panic disorder, and nine subjects with panic disorder but without alcoholism have been studied. Results indicate that there is a statistically significant difference in the behaviorally-induced effects of procaine between the groups. Panic disorder patients were more likely to experience a panic attack following procaine than were alcoholics without panic disorder or controls. There was no significant difference between groups in vagal tone or maximum heart rate following procaine. There were no statistical differences between the four groups for procaine-induced changes, in cortisol, ACTH, epinephrine, norepinephrine, or prolactin.

Significance to Biomedical Research and the Program of the Institute:

Our results suggest procaine may be a useful probe to characterize CNS panic differences between subjects with panic disorder and controls. The fact that the groups differed in their behavior responses but not according to their biochemical response suggests that the mechanism(s) responsible for procaine-induced panic is not directly linked to the systems governing the neuroendocrine parameters we studied. The issue of kindling will be addressed when the EEG data are analyzed.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AA 00278-05 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Behavioral and Physiological Effects of 2-Deoxyglucose Infusions**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. George Senior Clinical Investigator LCS, NIAAA

Others: M. Eckardt Senior Investigator LCS, NIAAA  
M. Linnoila Scientific Director NIAAA

COOPERATING UNITS (if any)

Veteran's Hospital, Washington, DC (T. Alim, T. Lindquist)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Science

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

2-deoxyglucose (2-DG) is a glucose analog which competitively inhibits glucose-6-phosphate dehydrogenase and leads to intracellular glucoprivation. In previous studies, 2-DG has been used as a stressor to activate both the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic adrenal axis and stimulate the appetitive centers of the hypothalamus. Our interest in this paradigm was generated by the clinical observation that alcoholics frequently consume increased amounts of carbohydrates following cessation of drinking. In order to explore possible hypothalamic abnormalities in patients with alcoholism, we administered 2-DG to abstinent alcoholics and measured the resulting behavioral and physiological changes arising from the 2-DG challenge. We postulated that a 2-DG induced glucoprivic response would give rise to both neuroendocrine and behavioral changes that might elucidate mechanisms of alcohol's action in the hypothalamus. Analysis of the results shows alcoholics consume less calories than controls following both 2-DG and placebo administration. Conversely, alcoholics show an exaggerated hypothalamic, ACTH response to glucoprivation.

Project Description:Investigators:

D. George	Senior Clinical Investigator	LCS, NIAAA
T. Alim	Staff Physician	Veteran's Hospital, Washington, DC
M. Eckardt	Senior Investigator	LCS, NIAAA
T. Lindquist	Biologist	Veteran's Hospital, Washington, DC
M. Linnoila	Scientific Director	NIAAA

Objectives:

The study is designed to develop a specific chemical-stress paradigm that will allow us to study the effects of glucoprivation on behavior and neuroendocrine control. Clinical experience suggests that upon cessation of ethanol ingestion many alcoholics exhibit an increase in carbohydrate ingestion. In this regard, since alcohol is also a carbohydrate, it is possible that alcohol may be substituted for conventional carbohydrates. In this protocol, we are interested in determining whether intracellular glucoprivation may serve as an internal "cue" that triggers an "urge to drink" alcohol.

Methods Employed:

Prior to participation in the study, all subjects are carefully evaluated to assure they are in good physical health and conform to predetermined diagnostic criteria. During the study, subjects receive placebo and two intravenous doses of 2-DG in random order. The subjects are carefully monitored for drug-induced changes in physical measures (pulse, blood pressure, vagal tone), mood, and hormones (prolactin, ACTH, cortisol).

Major Findings:

Data have been collected on 26 alcoholics and 15 normal volunteers. Minimal effects were observed following administration of the 12.0 mg/kg of body weight dose 2-DG. Following 25.0 mg/kg, alcoholics showed both exaggerated ACTH and cortisol responses and greater increases in caloric intake when compared with controls. Although anxiety, desire to consume alcohol, plasma progesterone, and sympathetic and adrenal medullary activity all increased following 2-DG, these responses did not differ between alcoholics and controls.

Significance to Biomedical Research and the Program of the Institute:

In this protocol, we explore the possibility that changes in intraneuronal glucose concentrations may trigger an urge to consume alcohol in patients with alcoholism. The present findings suggest certain specificity for the exaggerated hypothalamic and adrenocortical responses to mild glucoprivic stress in three week-abstinent alcoholics.



This finding is particularly interesting given a literature that states that alcoholics frequently drink to alleviate stress. Additional studies are now planned to evaluate hypothalamic function in alcoholics who have been abstinent for at least six months.

Publications:

George DT, Lindquist T, Alim T, Flood M, Eckardt MJ, Linnoila M. Abstinent alcoholics exhibit an exaggerated stress response to 3-deoxy-D-glucose challenge, Alcohol Clin Exp Res 1994;18:685-91.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00286-05 LCS

October 1, 1993 to September 30, 1994

Psychobiology of Alcoholism in Women

PI: D. George Senior Clinical Investigator LCS, NIAAA

Others: M. Linnoila Scientific Director NIAAA

P. Ragan Senior Clinical Associate LCS, NIAAA

COOPERATING UNITS (if any)  
None.

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Science

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

.50

PROFESSIONAL:

.50

OTHER:

.00

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies suggest that the pathophysiology of alcoholism may differ significantly between men and women. To explore this possibility, we have employed several pharmacological challenge paradigms to compare the behavioral and endocrine responses between male and female alcoholics. Preliminary analysis of our results shows that the m-chlorophenylpiperazine (m-CPP) gives rise to minimal behavioral effects in women. This corresponds to the responses obtained from Type I male alcoholics (late onset) demonstrated in a separate study. In contrast, only one of the women experienced a m-CPP induced "desire to drink" response demonstrated to occur in approximately 40% of the Type II (early onset) male alcoholics.

Project Description:Investigators:

D. George	Senior Clinical Investigator	LCS, NIAAA
M. Linnola	Scientific Director	NIAAA
P. Ragan	Senior Clinical Associate	LCS, NIAAA

Objectives:

In the past, many of the conclusions derived from alcohol research have either been based on male populations or have not specifically analyzed the data according to gender. However, it is becoming increasingly clear that alcoholism in women has a different clinical course compared to that in men. To begin with, women experience significantly higher blood levels after identical doses of alcohol per kilogram of body weight compared to men. Population studies show women develop problem drinking later in life compared to men, while at the same time experiencing many of the complications of alcoholism, i.e., cirrhosis and malnutrition, at approximately the same age as men. Women need consume only half the number of grams of ethanol per day as that of men to be at risk for liver cirrhosis. Women alcoholics have similar amounts of brain shrinkage compared to male alcoholics as measured by computerized tomography, but with significantly shorter alcohol exposure histories. These differences highlight the need to study alcoholism specifically in women. In males, m-chlorophenylpiperazine (m-CPP) gives rise to a "high" and an "urge to drink" in approximately 40% of the Type II alcoholics, but rarely in Type I alcoholics. We are investigating effects of m-CPP in female alcoholics.

Methods Employed:

Prior to participation in each study, all subjects were carefully screened to assure good physical health and conformance to predetermined diagnostic criteria. The paradigm of m-CPP challenge has been used as follows: 0.08 mg/kg of m-CPP or saline is infused in double-blinded fashion two days apart. Women alcoholics have disrupted menstrual cycles; however, it is important to do the infusions at comparable times across all subjects. Controls are age-matched nonalcoholic women. The goal is to perform the infusions during the early follicular phase in all subjects when estrogens are at their lowest ebb. Dependent measures include prolactin, ACTH, cortisol, body temperature, and behavioral measures (drug side effects profile). In a subgroup of the women controls, m-CPP infusions are done during both early follicular and late luteal phases of different menstrual cycles in order to measure responses (dependent variables) with this paradigm under these contrasting conditions. In a subgroup of women alcoholics, CSF samples are obtained prior to the m-CPP infusion in order to compare 5-HIAA levels to the serotonin agonists responses.

Major Findings:

To date, we have administered m-CPP in a double-blinded fashion to eight women alcoholics and two controls. Behaviorally, the women described mild anxiety symptoms similar to those described by Type I male alcoholics. Only one woman described an "urge to drink" which has been described by approximately 40% of the Type II male alcoholics.

Significance to Biochemical Research and the Program of the Institute:

By comparing parameters related to serotonin function in men and women, we hope to gain new insights into the pathogenesis of alcoholism in women.

Z01 AA 00286-05 LCS

Publications:

None.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00068-03 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

CNS Serotonin and the Regulation of Peripheral Glucose Metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. Eskay	Research Physiologist	LCS, NIAAA
Others:	T. Chautard	Visiting Fellow	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA
	M. Torda	Visiting Scientist	LCS, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry and Neuroendocrinology

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

1.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Increased alcohol preference and consumption, depressed mood, and impulsive aggression are thought to be linked in part through decreased central serotonergic (5-HT) activity. In agreement with this postulate, certain agents which increase central serotonergic neurotransmission (5-HT precursors, 5-HT uptake inhibitors, 5-HT receptor agonists) attenuate ethanol intake, improve memory function in intoxicated patients, and may improve memory functions in patients with Korsakoff's psychosis. Recently, a possible pattern of atypical glucose metabolism has emerged in alcohol abusing, impulsive violent offenders with apparent central serotonergic dysfunction. In a group of impulsive offenders, hypoglycemia was noted during an oral glucose tolerance test. The observed hypoglycemia was possibly due to increased insulin secretion. It is possible that a relative hypoglycemic state or abnormal insulin levels may contribute to violent, aggressive behavior in violent offenders with apparently reduced central 5-HT activity; however, this hypothesis awaits substantially more scientific verification. Although appropriate animal studies have not been performed which demonstrate a cause and effect relationship between altered central serotonin activity and abnormal glucose metabolism, there is overwhelming evidence that appropriate glucose levels are maintained through a complex feedback system which involves the sympathoadrenalmedullary system through the glucose mobilizing hormone epinephrine and the endocrine pancreas via insulin and glucagon secretion.

Project Description:Investigators:

R. Eskay	Research Physiologist	LCS, NIAAA
T. Chautard	Visiting Fellow	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
M. Torda	Visiting Scientist	LCS, NIAAA

Objectives:

The objectives of these studies are to demonstrate that if central serotonin-containing neurons are to be considered a significant central cell type which either respond directly to energy substrate changes or act as transneuronal mediators of such changes, the distribution of central 5-HT cell bodies and their projections or nerve terminal should be consistent with the purported areas of the central nervous system (CNS), particularly the ventral hypothalamic region, which historically have been shown to play a role in the control of feeding behavior and energy metabolism. Secondly, the central administration of selective serotonergic agonists and/or antagonists to intact animals or animals with discrete anatomic lesions or widespread 5-HT neuronal destruction should evoke consistent acute changes in either insulin which tends to inhibit glucose mobilization from the liver or glucagon, epinephrine, or norepinephrine, which promote hepatic glucose output.

Methods Employed:

In an attempt to understand the possible involvement of central serotonin in the regulation of peripheral glucose metabolism, a series of studies are underway in unrestrained, conscious adult male rats containing an indwelling jugular cannula and a chronic intracerebroventricular cannula for the peripheral and/or central administration of serotonin agonists/antagonists and to profile the changes in plasma glucose, insulin, and C-peptide. Representative members of the various serotonin receptor subtype specific agents (5-HT 1A, 1B, 1C, 1D; 5-HT 2; 5-HT 3) are being given centrally and/or peripherally and the changes as outlined previously will be followed.

Major Findings:

No new major findings.

Significance to Biomedical Research and the Program of the Institute:

The continued exploration of the involvement of serotonin in the central regulation of peripheral glucose metabolism will further our understanding of the role that central serotonin may play in the etiology and/or continuation of alcoholism and certain forms of depression and violent behavior. These studies in turn may hasten the development of effective therapeutic approaches and agents to treat these seemingly diverse dysfunctions.

Proposed Course:

The approaches and methods, as outlined, will continue with the intent to establish the CNS anatomic site and or serotonergic pathways and receptor subtypes which mediate our observations to date.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00287-04 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Stress Axis, Immune System-Derived Cytokines and Ethanol

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. Eskay Research Physiologist LCS, NIAAA

Others: T. Chautard Visiting Fellow LCS, NIAAA

M. Torda Visiting Scientist LCS, NIAAA

COOPERATING UNITS (if any)

Laboratory of Cell Biology, NIMH (M. Palkovitz, E. Mezey)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry and Neuroendocrinology

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Consumption of ethanol (Et) alters certain regulatory aspects of the hypothalamic-pituitary-adrenal axis (HPAA). Because the integrity of this system depends on the coordinated synthesis and secretion of specific regulatory substances at the hypothalamic (e.g., corticotropin-releasing hormone (CRH); vasopressin (AVP); biogenic amines), pituitary-gland (e.g., beta endorphin (BE); ACTH) and adrenal gland (e.g., catecholamines; glucocorticoids) level, we have been evaluating the impact of Et at each level of the HPAA. Activation of the HPAA or hypercortisolism accompanies both short- and long-term consumption of Et and the Et withdrawal syndrome. Alcoholics often present with a pseudo-Cushing's syndrome in which some 17-40% of alcoholics do not respond to the dexamethasone suppression test during the first week of abstinence. Since a relative state of elevated glucocorticoids (chronic continuous or chronic intermittent) can lead to neural changes and even cell death, particularly in the hippocampus, the progressive loss of cognitive capacity in many alcoholics may indeed be due in part to hypercortisolemia and subsequent irreversible neural damage in the hippocampus and other areas of the central nervous system. Furthermore, armed with the concept of the bidirectional communication between the HPAA and the immune system, we are exploring whether or not certain immune system-derived cytokines may be ameliorating or accelerating neural death through endocrine or paracrine actions. Certainly cytokines stimulate diverse cell types in an attempt to repair cellular damage through intracellular signal amplification which could in concert with Et and glucocorticoids overstimulate selected neural populations leading to their demise.

Project Description:Investigators:

R. Eskay	Research Physiologist	LCS, NIAAA
T. Chautard	Visiting Fellow	LCS, NIAAA
E. Mezey	Guest Researcher	LCB, NIMH
M. Palkovitz	Visiting Scientist	LCB, NIMH
M. Torda	Visiting Scientist	LCS, NIAAA

Objectives:

The ongoing aims of these studies are: (1) to profile ethanol-induced changes in the HPAA and immune system with a particular emphasis on the immune system derived cytokines which alter stress axis function; (2) to understand the sequence of events in these systems from membrane-receptor to intracellular-messenger systems to gene activity to physiological responses; (3) to determine the concentration-dependent and time-dependent effects of ethanol (Et) on the various cellular events as outlined in 1 and 2; and (4) to elucidate the consequences of ethanol's indirect effects on the HPAA and immune system, which may be mediated through elevated glucocorticoids and altered cytokine levels, respectively.

Methods Employed:

In an attempt to understand the precise mechanism or site of Et's activation of the HPAA, a series of studies were initiated in unrestrained, conscious adult male rats containing an indwelling jugular cannula for blood sampling and an indwelling intragastric cannula for ethanol administration. Animals received either a single infusion of a moderately high dose of ethanol (3.2gm/kg) or were continuously (0.5, 1, 3, 7 days) exposed to an ethanol/liquid diet via intragastric intubation in which blood ethanol levels were maintained between 150-300 mg% and compared to non-ethanol isocaloric control animals.

Major Findings:

No new major findings.

Significance to Biomedical Research and the Program of the Institute:

The continued exploration of the effects of Et on fundamental physiological systems, such as the stress axis and immune system, should provide additional understanding of multiple, ethanol-induced pathological perturbations of cellular events that are associated with alcoholism. This, in turn, will hasten the development of effective therapeutic agents to treat patients with alcohol-related dysfunctions.

Proposed Course:

Although activation or disruption of the HPAA axis through Et abuse results in hypercortisolemia and/or diminished cortisol rhythms, just how this contributes to CNS dysfunction is not clear. Since the hippocampus appears to be particularly sensitive to one of the consequences of Et consumption, namely elevated glucocorticoids, we will profile the effects of Et exposure on hippocampal function. Furthermore, the likelihood that cytokines like IL-1 and TNF participate in normal physiological events such as modulation of the stress axis, in addition to their known roles in inflammation and host defence, are being explored.

Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00069-03 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Na<sup>+</sup>, K<sup>+</sup>-ATPase Isoforms: Function and Regulation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: T. Foley IRTA Fellow LCS, NIAAA

Others: M. Linnoila Scientific Director NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry and Neuroendocrinology

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

While Na<sup>+</sup>, K<sup>+</sup>-ATPase activity has previously been found to be sensitive to ethanol, the role of this integral membrane enzyme as an effector of the neurophysiological actions of ethanol is poorly understood.

We have determined that a component of Na<sup>+</sup>, K<sup>+</sup>-ATPase-dependent synaptosomal K<sup>+</sup> uptake, which is defined as very sensitive (VS; IC<sub>50</sub> less than or equal to 10-7M) to inhibition by ouabain, is selectively activated by ethanol (EC<sub>50</sub>=3.3mM) under apparent resting conditions. Following increased intracellular Na<sup>+</sup> (i.e., depolarization), however, basal VS activity increases and the response to ethanol shifts to inhibition. This "state-dependent" effect of ethanol is mimicked by the steroid (ouabagenin) moiety of the glycoside ouabain. We are currently investigating the nature of the stimulatory and inhibitory ethanol "receptors."

In addition to the "state-dependent" actions of ethanol, it has also been observed that ethanol (20 mM) stabilizes diurnal variations in VS activity. The mechanism underlying the diurnal variations in VS activity is presently being examined.

Additional investigations are focusing on the developmental changes in the regulation of VS activity and the response of VS activity to ethanol. In general, the sensitivity of VS to stimulation and inhibition by ethanol decreases and increases, respectively, with age. Because stimulation of VS by ethanol disrupts the regulation of VS by monoamines, we propose that this action may have especially profound consequences in the developing brain.

Project Description:Investigators:

T. Foley  
M. Linnoila

IRTA Fellow  
Scientific Director

LCS, NIAAA  
NIAAA

Objectives:

The major objective of this project is to define the heterogeneity of Na<sup>+</sup>, K<sup>+</sup>-ATPase in the central nervous system so that the function and regulation of this enzyme can be examined more precisely under conditions relevant to the existence and treatment of alcoholism and other neuropathological disorders.

Methods Employed:

- (1) Subcellular fractionation of brain tissue
- (2) Radioligand tracer studies for the analysis of <sup>86</sup>Rb uptake, <sup>3</sup>H-ouabain binding and <sup>32</sup>P labeling
- (3) Immunoprecipitation
- (4) SDS-PAGE
- (5) UV-visible spectroscopy

Major Findings:

- (1) A component (VS) of the presumed alpha 1 isozyme of Na<sup>+</sup>, K<sup>+</sup>-ATPase is selectively activated by conditions which promote increased intracellular sodium.
- (2) The effect of ethanol (25 mM) on Na<sup>+</sup>, K<sup>+</sup>-ATPase activity is "state-dependent." Specifically, ethanol activates VS under resting conditions and inhibits VS activity under depolarized conditions.
- (3) Ouabagenin (1 uM), a Na<sup>+</sup>, K<sup>+</sup>-ATPase-specific steroid ligand, mimics the "state-dependent" actions of ethanol on Na<sup>+</sup>, K<sup>+</sup>-ATPase activity.
- (4) Ethanol (20 mM) stabilizes diurnal variations in VS activity.
- (5) The response of VS activity to ethanol is age-dependent. Activation of VS is most prominent in the developing brain and may dissociate VS from normal activation by monoamines.

Significance to Biomedical Research and the Program of the Institute:

This work establishes Na<sup>+</sup>, K<sup>+</sup>-ATPase as a possible mediator of the neurophysiological actions of low doses of ethanol. In addition, a basis for the rational design/identification of more specific ethanol-like modulators of Na<sup>+</sup>, K<sup>+</sup>-ATPase activity is provided.

Proposed Course:

- (1) Characterize the nature of the Na<sup>+</sup>, K<sup>+</sup>-ATPase "binding sites" for ethanol as well as for other mood-altering Na<sup>+</sup>, K<sup>+</sup>-ATPase ligands (i.e., lithium and rubidium).
- (2) Further define the basis for the diurnal variations in VS activity and the physiological significance of the stabilization by ethanol.

(3) Determine the structure of specific  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase steroid-based ligands required to mimic the actions of ethanol and other nonspecific mood altering  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase ligands (i.e., lithium and rubidium).

(4) Investigate the role of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase in the effects of ethanol on neuronal development.

Publications:

Foley TD, Linnoila M. Nanomolar concentrations of ouabain block ethanol-inducible  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in brain, Eur J Pharmacol, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00077-01 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

CNS Serotonin Activity, Anesthesia, and PET Scans in Rhesus Macaques

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.D. Higley	Senior Staff Fellow	LCS, NIAAA
Others:	P. Andreason	Senior Clinical Investigator	LCS, NIAAA
	D. Hommer	Section Chief	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA
	S. Shoaf	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS (if any)

Laboratory of Comparative Ethology, NICHD (S. Suomi, A. Dodson, T.S. King)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry and Neuroendocrinology

INSTITUTE AND LOCATION

Primate Unit, NIH Animal Center, PO Box 529, Building 112, Poolesville, MD 20837

TOTAL STAFF YEARS:

0.75

PROFESSIONAL:

0.25

OTHER:

0.50

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

To assess the role of serotonin functioning on excessive alcohol consumption and the related high levels of aggression and impaired impulse control, PET scans were performed on aggressive and nonaggressive rhesus macaques. Repeated CSF 5-HIAA samples were obtained from 10 young adult subjects and PET scans were performed on each. Subjects with diminished CSF 5-HIAA required more Phenobarbital anesthesia to produce unconsciousness than those with higher concentrations. There was a positive correlation between high CSF 5-HIAA concentrations and higher rates of deoxyglucose uptake in overall brain as well as the orbito-frontal region.

Our discovery of a primate model of excessive alcohol consumption allows the development of etiological models and specific treatments of human alcoholism. Through the use of the PET scan, we have begun to test specific hypotheses concerning the CNS functioning as an etiological mechanism producing human alcoholism. In terms of biological links to aggression, given the problems in studying aggression in humans, nonhuman primates are ideal candidates to test the correlation between serotonin functioning and aggression and to provide treatments for low serotonin-mediated aggression. In addition, our nonhuman primate model's use of carefully controlled rearing histories and genetic background allows tests of psychobiological mechanisms in a manner that is impossible in humans.



Project Description:Investigators:

J.D. Higley	Senior Staff Fellow	LCS, NIAAA
P. Andreason	Senior Clinical Investigator	LCS, NIAAA
A. Dodson	Veterinary Technician	LCE, NICHD
D. Hommer	Section Chief	LCS, NIAAA
T.S. King	Technician	LCE, NICHD
M. Linnoila	Scientific Director	NIAAA
S. Shoaf	Senior Staff Fellow	LCS, NIAAA
S. Suomi	Chief	LCE, NICHD

Objectives:

This project is concerned with the development of primate models investigating the role of central nervous system (CNS) serotonin functioning in excessive alcohol consumption, anxiety, and aggression. Special focus is placed on how early developmental experiences and genetic background shape CNS serotonin development and functioning. Such longitudinal studies are powerful tools for assessing the role of developmental experiences and genetic backgrounds and CNS functioning in excessive alcohol consumption and related problems.

Methods Employed:

Rhesus macaques are studied longitudinally from birth through adulthood either in naturalistic settings or in more controlled laboratory settings to assess their potential for excessive alcohol consumption, anxiety, and aggression. Those reared in the laboratory are selectively bred for excessive alcohol consumption or diminished CSF 5-HIAA concentrations. They are then reared either as a control group in a setting approximating normative rhesus macaque social organization or in anxiety-producing peer-only rearing groups. As young adults, they are provided free access to ethanol. Rates of alcohol consumption are linked to psychobiological variables and carefully controlled genetic and rearing backgrounds. In both feral and laboratory environments, as the young nonhuman primate develops, its behaviors are systematically sampled across major developmental phases. Differences in anxiety, aggression, social competence, and alcohol consumption are linked to individual differences in CNS functioning using measurements of hypothalamic-pituitary-adrenal activity and neurotransmitter system activity as assessed via CSF monoamine metabolite concentrations and Positron Emission Tomography (PET). Parents are studied to assess correlations between parental and offspring behaviors, CSF 5-HIAA, and PET scans. Because infants are reared either with or without parental exposure, genetic and environmental contributions can be assessed and understood.

Major Findings:

To assess the role of serotonin functioning on excessive alcohol consumption and the related high levels of aggression and impaired impulse control, PET scans were performed on aggressive and nonaggressive rhesus macaques. Repeated CSF 5-HIAA samples were obtained from 10 young adult subjects and PET scans were performed on each. Subjects with diminished CSF 5-HIAA required more Phenobarbital anesthesia to produce unconsciousness than those with higher concentrations. There was a positive correlation between high CSF 5-HIAA concentrations and higher rates of deoxyglucose uptake in overall brain as well as the orbito-frontal region.

Significance to Biomedical Research and the Program of the Institute:

Our discovery of a primate model of excessive alcohol consumption allows the development of etiological models and specific treatments of human alcoholism. Through the use of the PET scan, we have begun to test specific hypotheses concerning the CNS functioning as an etiological mechanism producing human alcoholism. In terms of biological links to aggression, given the problems in studying aggression in humans, nonhuman primates are ideal candidates to test the correlation between serotonin functioning and aggression and to provide treatments for low serotonin-mediated aggression. In addition, our nonhuman primate model's use of carefully controlled rearing histories and genetic background allows tests of psychobiological mechanisms in a manner that is impossible in humans.

Proposed Course:

Continued longitudinal assessment of young primates selectively bred for high or low CSF 5-HIAA concentrations using PET scans is planned as a major initiative over the next year.

Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00078-01 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of Stress on Imipramine Pharmacokinetics in Rhesus Macaques

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.D. Higley	Senior Staff Fellow	LCS, NIAAA
Others:	M. Hasert	IRTA Fellow	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA
	S. Shoaf	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS (if any)

Laboratory of Comparative Ethology, NICHD (S. Suomi, A. Dodson, T.S. King)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry and Neuroendocrinology

INSTITUTE AND LOCATION

Primate Unit, NIH Animal Center, PO Box 529, Building 112, Poolesville, MD 20837

TOTAL STAFF YEARS:

1.75

PROFESSIONAL:

0.50

OTHER:

1.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This study was suspended following the first treatment due to the low concentrations of imipramine and its metabolites that were obtained.

Project Description:Investigators:

J.D. Higley	Senior Staff Fellow	LCS, NIAAA
A. Dodson	Veterinary Technician	LCE, NICHD
M. Hasert	IRTA Fellow	LCS, NIAAA
T.S. King	Technician	LCE, NICHD
M. Linnoila	Scientific Director	NIAAA
S. Shoaf	Senior Staff Fellow	LCS, NIAAA
S. Suomi	Chief	LCE, NICHD

Objectives:

The major objective of this project is to determine the effect of changes in the sympatho-adrenal axis on the pharmacokinetics of antidepressant medications, so as to assess the potential of antidepressant treatment of excessive alcohol consumption.

Methods Employed:

Nine rhesus macaques were housed in a social group. They were trained to enter two same-sized cages connected by a mesh tunnel. While the subjects were in the tunnel they were given a treat. During the actual study the treat was loaded with 20 mg/kg imipramine (IMI) as imipramine pamoate. A crossover design was employed and the subjects were assigned to one of two groups of four or five subjects. For the first 21 day period, while the four individuals in one group were housed together in their home cage, the five individuals in the other group were subjected to stress in the form of a five day social separation followed by two days of home cage reunion. They were given 20 mg/kg IMI on each day. Blood samples were taken two times per week so that steady-state concentrations of IMI and its metabolites, desipramine (DMI), 2-hydroxyimipramine (2OHIMI), and 2-hydroxydesipramine (2OHDMI), could be determined. Cerebrospinal fluid was sampled at the same time to be analyzed for corticotropin-releasing factor (CRF) and neurotransmitter metabolites, MHPG, HVA, 5-HIAA, DOPAC, as measures of stress. On the 21st day, each monkey was given an intravenous dose of 14C-IMI, 20 mg/kg. Four blood samples were taken over the next 24 hours. The monkeys were then housed with their social group for four weeks to allow all imipramine/metabolites to be eliminated.

In the crossover, the monkeys originally kept together as a social group were to undergo the social separation paradigm while the other group was left intact. This allowed for each monkey to act as its own control.

Plasma concentrations of IMI, DMI, 2OHIMI, and 2OHDMI were determined by HPLC with ultraviolet detection. Steady-state concentrations following oral dosing were compared with elimination rates determined from the clearance of [14C] IMI.

Major Findings:

Nine monkeys, five stressed by social separation, were given 20 mg/kg imipramine, orally, once per day for three weeks, a dose that generally produces therapeutic blood concentrations in humans. Steady-state plasma concentrations of imipramine and its metabolites, DMI, 2OHIMI, and 2OHDMI, were well below 20 ng/ml for all animals.

14C-IMI concentrations were below detectable limits (1 ng/ml) in six hours or less.

No differences in steady-state concentrations were observed between groups.



Concentrations were much below reported therapeutic concentrations. The bioavailability of IMI following oral administration is extremely low.

Significance to Biomedical Research and the Program of the Institute:

It has been observed that changes in the sympatho-adrenal function, such as those induced by stress, may increase the rate of metabolism of antidepressant medications. If the clearance of these compounds is altered by stress then the traditional dosing regimens for individuals that are suffering from psychopathological disorders may need to be altered according to the stress being experienced by that individual.

Proposed Course:

This study will be terminated. Doses needed to produce therapeutic concentrations in rhesus macaques are not practical. Therapeutic concentrations of IMI are not attainable because of the high rate of first pass elimination and the limited palatability of the imipramine pamoate formulation.

Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00079-01 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Psychobiology of Antisocial Behavior, Social Competence, & Psychosomatic Health

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.D. Higley	Senior Staff Fellow	LCS, NIAAA
Others:	L. Akhtar	Chemist	LNG, NIAAA
	D. Goldman	Chief	LNG, NIAAA
	A. Lilly	IRTA Fellow	LCS, NIAAA
	S. Lindell	IRTA Fellow	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA
	D. Nielsen	Senior Staff Fellow	LNG, NIAAA
	S. Shoaf	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS (if any)

Laboratory of Comparative Ethology, NICHD (S. Suomi, A. Dodson, T.S. King);  
Laboratory Animal Breeders Services, Yemassee, SC (P. Mehlman, D. Taub); U of  
California, Dept. of Psychiatry (R. Poland)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry and Neuroendocrinology

INSTITUTE AND LOCATION

Primate Unit, NIH Animal Center, PO Box 529, Building 112, Poolesville, MD 20837

TOTAL STAFF YEARS:

3.7

PROFESSIONAL:

1.2

OTHER:

2.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

During the past year, our research included studies of: (1) investigation of behaviors that laboratory work has shown are correlated with excessive alcohol consumption, including aggression, impulsivity, and antisocial personality; (2) psychobiological mechanisms underlying individual differences in these behaviors; (3) 49 adolescent female rhesus macaques that were removed from the island to investigate the role of temperament, psychosocial health, and competent social behavior; and (4) molecular biology to assess genetic mechanisms underlying interindividual differences in CSF 5-HIAA concentrations.

Project Description:Investigators:

J.D. Higley	Senior Staff Fellow	LCS, NIAAA
L. Akhtar	Chemist	LNG, NIAAA
A. Dodson	Veterinary Technician	LCE, NICHD
D. Goldman	Chief	LNG, NIAAA
T.S. King	Technician	LCE, NICHD
A. Lilly	IRTA Fellow	LCS, NIAAA
S. Lindell	IRTA Fellow	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
P. Mehlman	Facility Scientist	LABS
D. Nielsen	Senior Staff Fellow	LNG, NIAAA
R. Poland	Professor	UCLA, CA
S. Shoaf	Senior Staff Fellow	LCS, NIAAA
E. Singley	Chemist	LCS, NIAAA
S. Suomi	Chief	LCE, NICHD
D. Taub	Facility Scientist	LABS
K. Zajicek	Technician	LCE, NICHD

Objectives:

This project is concerned with the development of primate models investigating excessive alcohol consumption, anxiety, and aggression. Principal research centers on the effect of developmental experience and genetic influences on alcohol consumption, anxiety, immunological functioning, aggression, and antisocial temperament. Assessments of sexual behavior, rates of pregnancies, surviving offspring, and molecular genetic probes are used to assess genetic effects.

Methods Employed:

Rhesus macaques are studied longitudinally from birth through adulthood in naturalistic settings to assess their potential for excessive alcohol consumption, anxiety, and aggression. As the young nonhuman primate develops, its behaviors are systematically sampled across major developmental phases. Differences in anxiety, aggression, social competence, and alcohol consumption are linked to individual differences in central nervous system (CNS) functioning using measurements of hypothalamic-adrenal-pituitary and neurotransmitter system activities as assessed via CSF monoamine concentrations. Because highly aggressive monkeys are difficult to maintain in the laboratory, few are kept in most colonies. This paucity of aggressive laboratory dwelling nonhuman primates has made the Morgan Island project crucial to identifying highly aggressive and submissive monkeys from which we can obtain CSF and blood plasma samples. To directly assess behavioral differences, the Morgan Island free-ranging subjects from which we obtained CSF samples were radio collared and marked for ease of location and identification. Some subjects are removed from the island environment to assess in carefully controlled laboratory environments where temperament ratings, CSF, and blood samples can be obtained to assess temperament, CNS, and immunological functioning as predictors of reintegration into new social groups and the acquisition of social competence.

The relationship between serotonin and social behavior in laboratory and feral-living nonhuman primatesSerotonin, Testosterone and Aggression

Our previous results demonstrated that subjects in the wild, who had more severe scars and wounds had low CSF 5-HIAA. As a replication of our earlier findings, Mr. Lindell, one of our IRTA trainees, continued our investigation of scars and wounding, rating two different cohorts for aggressiveness. We found that interindividual differences in wounds and scar location and frequencies were predictive of CSF 5-HIAA concentrations obtained within three months of the scar

and wound count. Individuals possessing more wounds and scars have low CSF 5-HIAA. Following CSF sampling, behavior was directly sampled from these subjects. Excessive and inappropriate aggression was negatively correlated with CSF 5-HIAA. In a study performed in collaboration with Dr. Russel Poland of UCLA, we replicated these findings. As in our previous study, we found that low concentrations of CSF 5-HIAA were correlated with high levels of inappropriate, excessive aggression. Interestingly, further analysis indicated that CSF 5-HIAA was not correlated with overall rates of aggression and submission. CSF testosterone concentrations, on the other hand, were positively correlated with overall rates of aggression and increased submission, but not with excessive inappropriate aggression.

#### Serotonin and Impulsivity

Evidence indicated that the inappropriate behaviors seen in subjects with low CSF 5-HIAA resulted from deficits in impulse control. Studies last year indicated that subjects with low CSF 5-HIAA were more likely to make spontaneous, dangerous leaps from tree-tops. This finding was replicated this year, and we also found that the young males with low CSF 5-HIAA were more likely to jump into the traps used to capture subjects. These later findings and those described in annual report Z01 AA 00277-04 LCS, showing excessive alcohol consumption in subjects with low CSF 5-HIAA, suggest that the genesis of dysfunctional behaviors in subjects with low CSF 5-HIAA may be due to impaired impulse control.

Studies were also initiated to assess how subjects with low CSF 5-HIAA and dysfunctional impulse control integrate and maintain themselves in their society. Our results showed that individuals with low CSF 5-HIAA and individuals who made spontaneous, dangerous leaps exhibited antisocial tendencies, spending less time in close proximity to other subjects when they did interact, their social interactions brusque and unsophisticated. While in natural settings all males leave their natal troops following childhood, we found that subjects with low CSF 5-HIAA left their troops at an earlier age, when they are smaller in size, and less likely to have friends in other troops with whom they can form coalitions to integrate themselves into the new troops. Perhaps as a result, of the first seven subjects that have died either from aggressive encounters or from illnesses, five were below the overall population mean in CSF 5-HIAA, and they possessed lower 5-HIAA than the overall population.

Ms. Lilly, our IRTA Fellow, played an important role by beginning investigations of a relatively large female cohort. A major initiative of the past year was an investigation of 48 females that have been removed from the island and placed in single cages prior to being placed into social groups. Quarterly CSF and blood samples were obtained from each of the subjects. Daily temperament profiles, gross behavioral samples including hostility and aggression to the experimenter, fearfulness, and activity were obtained. Health assessments were obtained daily including ratings of constipation and diarrhea, allergic reactions, and illnesses. Careful and systematic assessments for parasitic infestation were made with plans to link them with severity of symptomatology. Data analysis has only begun on these subjects; however, initial findings indicate that like our island males, interindividual differences in CSF 5-HIAA were highly stable, with island values predicting single-caged samples and single-caged samples showing stability from quarter to quarter. Blood chemistry and immune measures obtained from the blood samples indicated trait-like interindividual blood chemistry levels and immune responses, with red blood cell counts, hemoglobin, hematocrits, mean corpuscular volume, white blood cell counts, neutrophils, lymphocytes, platelets, absolute numbers of CD4 and CD8, CD4/CD8 ratio, CD20, CD2, HVA, correlated from quarter to quarter.



Significance to Biomedical Research and the Program of the Institute:

Our discovery of a primate model of excessive alcohol consumption allows the development of etiological models and specific treatments of human alcoholism. Aggression, antisocial personality, and impulse deficits are major problems in humans who abuse alcohol and suffer from alcoholism. These problems are difficult to study in laboratory environments where aggressive and impulsive individuals are frequently culled to prevent injuries to the other less aggressive subjects. Because this is a natural population and aggressive animals are not culled, the Morgan Island project is crucial to maintaining a relatively large subpopulation of aggressive, impulsive subjects. In terms of biological links to aggression, given the problems in studying aggression in humans, nonhuman primates are ideal candidates to test the correlation between serotonin and aggression and to provide treatments for low serotonin-mediated aggression.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00277-06 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nonhuman Primate Models of Alcohol Consumption and Aggression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.D. Higley	Senior Staff Fellow	LCS, NIAAA
Others:	L. Akhtar	Chemist	LNG, NIAAA
	D. Goldman	Chief	LNG, NIAAA
	M. Hasert	IRTA Fellow	LCS, NIAAA
	S. Lindell	IRTA Fellow	LCS, NIAAA
	M. Linnoila	Scientific Director	NIAAA
	D. Nielsen	Senior Staff Fellow	LNG, NIAAA
	S. Shoaf	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS (if any)

Laboratory of Comparative Ethology, NICHD (S. Suomi, A. Dodson, T.S. King, W. Thompson, C. Zajicek); University of California, Department of Psychiatry (R. Poland)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry and Neuroendocrinology

INSTITUTE AND LOCATION

Primate Unit, NIH Animal Center, PO Box 529, Building 112, Poolesville, MD 20837

TOTAL STAFF YEARS:

6.75

PROFESSIONAL:

1.25

OTHER:

5.50

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

During the past year, our research included studies of: (1) etiological mechanisms underlying individual differences in alcohol consumption, including the effects of age, alcohol exposure setting, sex, and paternal genetic contributions; (2) the effect of early experience on the development of the nervous system and on the acquisition of social competence and aggressive behaviors; (3) the relationship between serotonin (5-HT) and aggression; and (4) developmental patterns of rhesus macaques selectively bred for extremes in CSF 5-HIAA. A major part of the past year has involved the continued investigation of the infants (now in their late childhood and early adolescence) that were selectively bred for extremes in CSF 5-HIAA concentration. Both the parents and the infants have been carefully assessed to investigate parental genetic contributions to aggression and alcohol consumption.

Project Description:Investigators:

J.D. Higley	Senior Staff Fellow	LCS, NIAAA
L. Akhtar	Chemist	LNG, NIAAA
A. Dodson	Veterinary Technician	LCE, NICHD
D. Goldman	Chief	LNG, NIAAA
M. Hasert	IRTA Fellow	LCS, NIAAA
T.S. King	Technician	LCE, NICHD
S. Lindell	IRTA Fellow	LCS, NIAAA
M. Linnola	Scientific Director	NIAAA
P. Mehlman	Facility Scientist	LABS
D. Nielsen	Senior Staff Fellow	LNG, NIAAA
R. Poland	Professor	UCLA
S. Shoaf	Senior Staff Fellow	LCS, NIAAA
E. Singley	Chemist	LCS, NIAAA
W. Thompson	IRTA Fellow	LCE, NICHD
K. Zajicek	Technician	LCE, NICHD

Objectives:

This project is concerned with the development of primate models investigating excessive alcohol consumption, anxiety, and aggression. Principal research centers on the effect of developmental experience and genetic influences on alcohol consumption, anxiety, and aggression. This research is performed by investigating developmental pathways in nonhuman primates exhibiting temperamental differences, the effects of early rearing, genetic background, and the stability of interindividual differences in behavioral styles and central nervous system function. Behavioral, hormonal, and neurotransmitter measurements, genetic probes, and psychopharmacological manipulations are its major tools.

Methods Employed:

Rhesus macaques are studied longitudinally from birth through adulthood to assess their potential for excessive alcohol consumption, anxiety, and aggression. Subjects are selectively bred for excessive alcohol consumption or diminished CSF 5-HIAA concentrations. They are then reared either as a control group in a setting approximating normative rhesus macaque social organization or in anxiety producing peer-only rearing groups. As young adults, they are provided free access to ethanol. Rates of alcohol consumption are linked to psychobiological variables and carefully controlled genetic and rearing backgrounds. As the young nonhuman primate develops, its behaviors are systematically sampled across major developmental phases. Differences in anxiety, aggression, social competence, and alcohol consumption are linked to individual differences in CNS functioning using measurements of hypothalamic-adrenal-pituitary activity and neurotransmitter system activity as assessed via CSF monoamine metabolite concentrations. Parents are studied to assess correlations between parental and offspring behaviors. Because infants are reared either with or without parental exposure, genetic and environmental contributions can be assessed and understood.

Major Findings:

Etiological mechanisms underlying individual differences in alcohol consumption in rhesus macaques, with an emphasis on paternal genetic contributions  
In previous studies of rhesus macaques, we found that early experiences influence later alcohol consumption in young adults, with rearing conditions such as peer-rearing increasing anxiety and fearfulness, which produces excessive alcohol consumption. Further studies during the past year show that the social setting in which alcohol is dispensed plays a differential role in the rate of alcohol consumption for males and females. Males consume more than females when they

drink in mixed and same sex pairs, but not when they are in less intimate larger group settings, but in larger group settings, male and female macaques consume alcohol at similar rates. Adult male alcohol consumption has also been assessed in single cage settings. When males drink alone, they consume at levels almost identical to when drinking in pairs, and these levels are significantly higher than when the males are studied in larger social groups. One important new finding is that levels of CSF 5-HIAA show a negative correlation with alcohol consumption rates. This correlation was present in both males and females.

#### Developmental patterns of rhesus macaques selectively bred for extremes in CSF 5-HIAA

On the basis of our findings showing genetic contributions to excessive alcohol consumption and interindividual differences in CSF 5-HIAA, a major initiative during the past year involved continued selective breeding of nonhuman primates for extremes in alcohol consumptions and CSF 5-HIAA concentrations. To date, 62 infants have been born from this initiative. Recent studies have shown that individuals differ in the temperature on the right and left sides of their body with temperatures on the left side of the body higher in individuals with high trait anxiety. In collaboration with Dr. Boyce, we assessed temperature differentials in these subjects using thermoscans of right and left eardrums. Individuals with higher cortisol concentrations and less activity during a social separation stressor exhibited higher left side temperatures, suggesting the potential to use left-right temperature differentials as risk markers for anxiety-mediated alcohol consumption. Parents have been carefully assessed to investigate potential parental genetic contributions to aggression and alcohol consumption. The parents were assessed before and during pregnancy for levels of aggression and concentrations of CSF monoamines and plasma hormone concentrations. Under the supervision of our IRTA Fellow Mariken Hasert, much of this year's focus has been on 20 of the mothers of these subjects. These 20 subjects were selected based on their aggressive nature and their known CSF 5-HIAA concentrations and have been followed over a three year period. They were taken from single cages or small social groups and placed in one of two identical social groups. Assessments of repeated CSF samples show a high degree of interindividual stability in CSF 5-HIAA, with a correlation coefficient averaging approximately 0.50 over a six month period. When samples obtained in the first year of the study were correlated with samples obtained in the third year, the average correlation accounted for over 50% of the variance in interindividual differences in CSF 5-HIAA. CSF 5-HIAA samples obtained prior to and during social grouping were predictive of a measure of social competence in rhesus macaques, social dominance ranking. Studies showed that having low CSF 5-HIAA prior to group formation resulted in low social dominance ranking. As with our males in feral habitats, females with low CSF 5-HIAA were also less likely to spend time in social interactions. Females with low CSF 5-HIAA were more likely to have been removed from their social groups for aggressiveness. In her master's thesis, which won a University award for excellence in research, and in a paper presented at the Society for Behavioral Medicine, which was given a special citation for excellence in research, Ms. Hasert demonstrated that diminished 5-HIAA, fearfulness, and increased social support were correlated with increased salivary immunoglobulin E, an immune measure that is typically found to be elevated in individuals with increased allergies.

#### The effect of early experience on the development of social competence and the nervous system

Comparisons between the peer- and mother-reared nonhuman primates indicate the importance of early experience on the developing primate nervous system. Paralleling the negative relationship between aggression and serotonin, data analyzed this year indicate that neonatal monkeys reared apart from their mothers exhibited lower CSF 5-HIAA concentrations than mother-reared subjects when they were 15 and 30 days of age. Interindividual differences in CSF 5-HIAA were highly stable from 15 days of age through six months of age. Continued study of these peer-reared subjects indicate that there are behavioral differences that parallel the central nervous system deficits. Studies from last year indicated



that during their second year of life after the mother-reared subjects are removed from their mothers and placed with the peer-reared subjects, the peer-reared subjects show evidence of diminished social competence, exhibiting decreased social dominance status, and spending less time in complex social play. Continued study of our first cohort, who are now three years of age (an age when aggression is first exhibited at levels approaching adult norms), subjects removed from their social group for treatment of wounds by veterinarian staff possessed lower than average CSF 5-HIAA.

Significance to Biomedical Research and the Program of the Institute:

Our discovery of a primate model of excessive alcohol consumption allows the development of etiological models and treatments of human alcoholism. Aggression, antisocial personality, and impulse control deficits are major problems in humans with alcoholism and alcohol abuse. In terms of biological links to aggression, given the problems in studying aggression in humans, nonhuman primates are ideal candidates to test the correlation between serotonin and aggression and to provide treatments for low serotonin-mediated aggression. In addition, as this nonhuman primate model is adopted by others, researchers will be able to carefully control rearing histories, data collection, and test psychobiological mechanisms in a manner that is impossible in humans.

Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00258-10 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Violent Behavior, Neurotransmitters, Glucose Metabolism, and Alcohol Abuse

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Linnoila Scientific Director NIAAA

Others: D. Goldman Chief LNG, NIAAA

COOPERATING UNITS (if any)

Department of Psychiatry, University of Helsinki (M. Virkkunen)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry and Neuroendocrinology

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.4

PROFESSIONAL:

0.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have investigated neurotransmitter metabolites and glucose metabolism in incarcerated violent offenders, arsonists, and healthy volunteers. We have found that low cerebrospinal fluid (CSF), 5-hydroxyindoleacetic acid (5-HIAA) concentrations, and hypoglycemia during oral glucose tolerance tests are associated with each other and impulsive violent acts and fire setting. In a follow-up study, we found that a low blood glucose nadir and low CSF 5-HIAA concentration were powerful predictors of recidivism among impulsive violent offenders and fire setters. We have documented a profound disturbance of day-night activity rhythms in alcoholic violent offenders with intermittent explosive disorder. The personality abnormalities associated with low CSF 5-HIAA in alcoholic violent offenders were found to be chronic irritability and psychasthenia. Sample collection for the molecular genetic family study on alcoholic violent offenders has been completed.

Project Description:Investigators:

M. Linnoila  
D. Goldman  
M. Virkkunen

Scientific Director  
Chief  
Senior Lecturer

NIAAA  
LNG, NIAAA  
U of Helsinki,  
Finland

Objectives:

The major objective of this project is to investigate psychobiological and genetic variables associated with impulsive and violent behaviors as well as alcohol abuse in humans.

Methods Employed:

Cerebrospinal fluid neurotransmitters and neurotransmitter metabolites have been quantified in samples obtained from violent offenders, arsonists, and healthy volunteers. The subjects have been administered oral glucose tolerance tests and MMPis, and they have undergone careful forensic psychiatric examinations. A follow-up study on recidivism has been completed using the criminal register of Finland to detect repeat crimes. Lymphocytes are currently being collected from violent offenders and their families to be used for genetic linkage studies.

Major Findings:

We have now documented a profound disturbance of day-night activity rhythms in alcoholic violent offenders with intermittent explosive disorder. The personality abnormalities associated with low CSF 5-HIAA in alcoholic violent offenders were found to be chronic irritability and psychasthenia.

Significance to Biomedical Research and the Program of the Institute:

Alcohol abuse is associated with a large proportion of violent offenses and arson. It has also been associated with low cerebrospinal 5-HIAA concentration. We have demonstrated clear associations between low cerebrospinal fluid 5-HIAA concentration, alcohol abuse, and violent behavior. Furthermore, we have found in arsonists associations between low glucose metabolism and alcohol abuse. These findings, if replicated by others, can form a rational basis for treatment interventions in these heretofore difficult-to-treat individuals.

Proposed Course:

We are collecting lymphocytes in a new sample of subjects to relate the described findings to possible Y-chromosome and serotonergic gene abnormalities. We have collected samples of blood relatives of the index individuals and appropriate controls to investigate the molecular genetics of these conditions.

Publications:

Virkkunen M, Kallio E, Rawlings R, Tokola R, Poland R, Guidotti A, Nemeroff C, Bissette G, Kalogeras K, Karonen S, Linnoila M. Personality profiles and state aggressiveness in Finnish violent offenders, impulsive fire setters, and healthy volunteers, Arch Gen Psychiatry 1994;51:28-33.

Virkkunen M, Rawlings R, Tokola R, Poland R, Guidotti A, Nemeroff C, Bissette G, Kalogeras K, Karonen S, Linnoila M. CSF biochemistries, glucose metabolism, and diurnal activity rhythms in violent offenders, impulsive fire setters, and healthy volunteers, Arch Gen Psychiatry 1994;51:20-28.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00233-12 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Family Studies of Alcoholism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G. Brown Clinical Director LCS, NIAAA

Others: I. Culver Psychologist LCS, NIAAA  
L. Doty Social Worker LCS, NIAAA  
S. Goodson Senior Staff Fellow LCS, NIAAA  
C. Jones Psychology Technician LCS, NIAAA  
V. Moore Social Worker LCS, NIAAA  
M. Trunzo Social Worker LCS, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Assessment & Biological Correlates

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

This project has been terminated.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00257-10 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Neuroendocrine Studies in Offspring of Familial Alcoholics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G. Brown Clinical Director LCS, NIAAA

Others: S. Goodson Senior Staff Fellow LCS, NIAAA  
M. Linnoila Scientific Director NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Assessment & Biological Correlates

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been terminated.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00276-06 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Psychobiology of Aggression and Suicide in Adults and Children

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G. Brown Clinical Director LCS, NIAAA

Others: P. Andreason Senior Clinical Investigator LCS, NIAAA  
S. Goodson Senior Staff Fellow LCS, NIAAA  
M. Linnoila Scientific Director NIAAA  
M. Trunzo Social Worker LCS, NIAAA  
W. Williams Senior Clinical Investigator LCS, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Assessment & Biological Correlates

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

1.0

CHECK APPROPRIATE BOXES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies that relate human aggression (including Disruptive Behavior Disorders in children) and suicide to various behavioral and biological factors have been ongoing. The most significant finding is a trivariate relationship among history of aggressive behavior, history of suicidal behavior, and low cerebrospinal fluid (CSF) 5-hydroxyindoleacetic acid (5-HIAA). The data indicate that certain aggressive, impulsive, and depressive characteristics in childhood are inversely related to CSF 5-HIAA measured during late adolescence. Family instability (particularly alcoholism in a parent) during childhood is also associated with an increased likelihood of aggressive and suicidal behavior in adolescence. Offspring of parents with major affective disorders are more likely to manifest suicidal behavior as adolescents than offspring of control parents. These data, along with the work of other investigators studying aggressive and depressive behavior in childhood, indicate the possibility of traits associated with disordered serotonin metabolism. Further, the consistent relationship between lower CSF 5-HIAA and suicidal behaviors and aggressive behaviors may indicate that some suicidal behaviors are a self-destructive manifestation of a more basic destructive (aggressive/impulsive) trait. A new focus has included the study of early onset substance abusers, both male and female with antisocial characteristics; these studies have now included American Indians and Finns. Related collaborations include PET studies and molecular genetics associated with serotonin metabolism.

Project Description:Investigators:

G. Brown	Clinical Director	LCS, NIAAA
P. Andreason	Senior Clinical Investigator	LCS, NIAAA
S. Goodson	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Scientific Director	NIAAA
M. Trunzo	Social Worker	LCS, NIAAA
W. Williams	Senior Clinical Investigator	LCS, NIAAA

Objectives:

The objective is to enhance knowledge of the central nervous system (CNS) of children, adolescents, and adults with reference to maturational changes, neuropsychiatric disorders, and molecular genetics as they relate to aggression and suicide. Alterations in neurotransmitter metabolism have been proposed for children and adolescents who have psychiatric diagnoses within the DSM-III-R Disruptive Behavior Disorder (DBD) group, viz., DSM-III-R diagnoses: Attention Deficit Disorder (ADD); Conduct Disorder (CD); and Oppositional Defiant Disorder (ODD). Searching for interrelationships between neurochemistry and repeated behavioral patterns including substance abuse may be more fruitful than searching for biochemical traits related to traditional diagnoses. Direct human data are important for assessing differences and similarities between humans and animals. Data relate CNS function to some behaviors associated with trait disorders, i.e., aggressive/impulsive (A/I), obsessive/compulsive, and substance abuse. A/I characteristics have been linked to a genetic contribution to suicidal behavior independent of psychiatric diagnoses. Animal data strongly suggest relationships between A/I behavior and neurotransmitters, particularly serotonin (5-HT). An ongoing interest is to extend studies of CNS amines into larger and more diverse groups.

Methods Employed:

In- and outpatient programs have involved children and adults (including American Indians and Finns). Offspring of normal parents and parents with a history of affective disorder have been studied; offspring of parents with a history of alcoholism and violence are also being studied. Children have been evaluated medically, psychiatrically, and psychometrically. Details can be found in NIMH protocols #85-M-115 and #79-M-123. Previous NIMH-NMNC studies have been described (Z01 MH 00092-11 BP). Among individuals incarcerated for murder on whom baseline cerebrospinal fluid (CSF) is available, psychological responses to the glucose tolerance test will be assessed by the Thematic Apperception Test; molecular genetics material are also being studied (Goldman, Linnoila et al.). These studies have been extended to children and families including the genetic regulation of tryptophan hydroxylase (TPH), a rate-limiting enzyme in the synthesis of 5-HT. PET studies in aggressive adults are ongoing (Andreason et al.). Further, areas of behavioral disturbance thought to be associated with 5-HT dysfunction in children and adolescents are being assessed, e.g., A/I behavior, sleep disturbance, suicidality, and substance abuse. Computer-assisted diagnostic interviews of children have been employed as an innovative way of assessing DBD. Computer-assisted SCID-II diagnoses are now being determined.

Major Findings:

A negative correlation exists between CSF 5-HIAA and childhood problems associated with ADD/CD, aggression, depression, and headaches. Lower CSF 5-HIAA has now been found in DBD vs. ODD children and predicts later A/I behavior as adolescents. In late adolescents, those with higher scores for A/I behaviors had greater mean scores on items related to family instability, lower levels of CSF, and a greater incidence of suicide attempts compared to those with lower scores for A/I behavior. Disturbed family history per se was not related to levels of

CSF 5-HIAA, possibly indicating various kinds of disturbed personality and behavior not of an aggressive and/or suicidal nature, which may not be associated with lower CSF 5-HIAA. These findings support an inverse relationship between A/I behavior (behavior thought to indicate dyscontrol and disinhibition as trait characteristics) and CSF 5-HIAA. Differences in molecular genetic determinants of TPH and monoamine oxidase now support these associations. Further, evidence seems to support A/I being more clearly related to 5-HT than a history of suicide attempts; suicidal behavior may be a form of aggressive behavior. Psychopathology in parents, particularly major affective disorder, is associated with an increase in suicidal behavior in adolescent offspring vs. offspring of control parents. Indications are that serotonergic traits, as described above, are more prevalent in the offspring of psychiatrically disturbed parents. Studies in American Indians indicate a high frequency of severe alcoholism and substance abuse with an early onset, particularly in males.

#### Significance to Biomedical Research and the Program of the Institute:

CNS functioning related to trait characteristics now receives similar attention as has been traditionally accorded to some major groups of psychiatric patients, viz., personality disorders, alcoholics, and borderlines vs. affective and schizophrenic disorders. Studies of animal models, ADD/CD, and prisoners suggest a relationship between central neurotransmitter systems and A/I behavior. These studies may identify contributory risk factors for antisocial, suicidal, and substance abuse behaviors and more effective treatment for those behaviors; these studies may also lead to a further understanding of the molecular genetics and the regulation of 5-HT metabolism.

#### Proposed Course:

The NIMH-NNMC collaborative project began in January 1973. Though data collection of the original NIMH-NNMC project is not now in process, this collaboration continues to be of mutual benefit to NIMH, NIAAA, and NNMC. Collaboration continues with NIMH (LDP): the project is proposed for two more years.

#### Publications:

Brown GL, Albaugh B, Robins R, Goodson SG, Trunzo M, Wynne DK, Goldman D. Alcoholism and substance abuse among selected Southern Cheyenne Indians, Culture, Medicine and Psychiatry 1993;16:531-42.

Brown GL, Goodson SG, Linnoila MI. Dopamine, serotonin and alcoholism. In: Chick J, Goeting NLM, MacLennan P, eds. Current Approaches - Alcohol Abuse, Duphar Medical Relations 1993: 15-21.

Brown GL. [Commentary]. In: Pohorecky LA and Brick J, eds. Symposium on alcohol and aggression, Suppl, Journal of Alcohol Studies, in press.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00022-01 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interaction of Chlorzoxazone and Caffeine in Smokers and Non-Smokers

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. Shoaf Senior Staff Fellow LCS, NIAAA

Others: D. George Senior Clinical Investigator LCS, NIAAA  
D. Herion Senior Staff Fellow LCS, NIAAA

COOPERATING UNITS (if any)

NIHCC (M. Flood, R. White)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Unit of Pharmacokinetic Studies

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This protocol has just recently been approved.

The high performance liquid chromatography methods for chlorzoxazone, caffeine, theobromine, theophylline, and paraxanthine have been developed and validated.

Project Description:Investigators:

S. Shoaf	Senior Staff Fellow	LCS, NIAAA
M. Flood	Dietician	CC, NIH
D. George	Senior Clinical Investigator	LCS, NIAAA
D. Herion	Senior Staff Fellow	LCS, NIAAA
R. White	RN	CC, NIH

Objectives:

The major objective of this project is to determine if chlorzoxazone (CZ) clearance measurements will be a suitable marker of cytochrome P4502E1 induction in alcoholics. CZ was initially reported to be exclusively metabolized to its 6-hydroxy metabolite by the cytochrome P4502E1 enzyme (Peter et al., Chem Res Toxicol 1990;3:566). Subsequent *in vitro* studies have reported that cytochrome P4501A1 may also be involved (Carriere, Chem Res Toxicol 1993;6:852). If we wish to use CZ as a marker of P4502E1 induction in alcoholics then the possible contribution of P4501A1 must be determined. P4501A1 is greatly induced by smoking. Therefore, the clearance of CZ would be faster in smokers compared to non-smokers if P4501A1 is contributing to CZ metabolism. Caffeine is metabolized by P4501A1 when present in concentrations of 1mM. CZ clearance in the presence of caffeine should be slowed if P4501A1 is contributing to CZ metabolism.

Methods Employed:

There will be four groups of 12 individuals studied. Male and female healthy volunteers who smoke (at least one pack per day for the last year) and do not smoke (not smoked for three years). A cross-over design will be employed. On one occasion the subject will be given an oral dose (7 mg/kg) of CZ. On a second day, the subject will first be given an oral dose (2 mg/kg) of caffeine. Two hours later the subject will be given an oral dose (7 mg/kg) of CZ. Blood samples will be drawn at 11 and 13 time points on the respective days and urine will be sequentially collected on the day that caffeine is given. Subjects will be asked to abstain from alcoholic beverages and caffeine-containing foods and beverages for three days prior to each drug administration. The clearance of CZ will be determined from the analysis of plasma samples by high performance liquid chromatography with UV detection. Caffeine and its metabolites, theobromine, theophylline, and paraxanthine, will also be determined by HPLC with UV detection. Data analysis will be done by ANOVA. Smokers vs. non-smokers, caffeine vs. no caffeine.

Major Findings:

None.

Significance to Biomedical Research and the Program of the Institute:

Many current liver function tests do not actually measure the ability of the liver to perform a metabolic function such as clearance of a xenobiotic compound. If chlorzoxazone is exclusively metabolized by the P4502E1 enzyme, then by doing sequential studies, we will be able to study how long ethanol-induced effects are sustained in alcoholics following ethanol withdrawal.

Proposed Course:

Male and female healthy volunteers, smokers and non-smokers, will be recruited.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00071-03 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

$\alpha$ -methyl-L-tryptophan as a Tracer of Brain Serotonin Synthesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. Shoaf Senior Staff Fellow LCS, NIAAA

Others: P. Andreason Senior Clinical Investigator LCS, NIAAA  
M. Eckardt Senior Investigator LCS, NIAAA  
M. Linnoila Scientific Director NIAAA

COOPERATING UNITS (if any)

Nuclear Medicine Department, CC (W. Eckelman, B. Schmall); Department of Medicine, U British Columbia, Canada (D. Doudet)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Unit of Pharmacokinetic Studies

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Monkeys have undergone the steady-state pharmacokinetic study and the plasma and brain samples are currently being analyzed.

L- $\alpha$ -methyltryptophan was obtained by chiral separation on an HPLC system. The solvent was removed by reinjection/fractionation over a C18 column using Meoh:Water as the mobile phase. The methanol was blown off and the remaining  $\alpha$ -methyl-tryptophan in water concentrated in a Speed-Vac.

Project Description:Investigators:

S. Shoaf	Senior Staff Fellow	LCS, NIAAA
P. Andreason	Senior Clinical Investigator	LCS, NIAAA
D. Doudet	Assistant Professor	Dept. of Medicine, U of British Columbia
M. Eckardt	Senior Investigator	LCS, NIAAA
B. Eckelman	Chief	NMD, CC
M. Linnoila	Scientific Director	NIAAA
B. Schmall	Chemist	NMD, CC

Objectives:

This research is designed to determine the rate of serotonin synthesis in various regions of the primate brain and is intended to provide the pharmacokinetic foundation for the development of a human positron emission tomography (PET) ligand (see annual report entitled "Brain Synthesis in Patients with Addictive and Aggressive Behavior"). It is planned that a-(11C)methyl-L-tryptophan will be administered in tracer quantities and the accumulation of radioactivity in brain tissue will be followed by use of PET. The use of a-(11C)MTP as a tracer for tryptophan (TP) requires that the pharmacokinetic, enzyme kinetic, and distribution differences of these compounds be known. Monkeys will be used to determine these differences in lieu of humans as there is no way to determine them without destructive sampling. Cold a-MTP will be administered as the kinetic/distributional differences will be much greater than the isotope effects.

Methods Employed:Determination of pharmacokinetic and distribution differences

Monkeys will be anesthetized and then administered a bolus dose of a-MTP. Arterial and venous blood samples will be taken and analyzed, by HPLC, to determine the kinetics of a-MTP. These data will then be used to determine an adjusted-rate infusion schedule that will allow steady-state concentrations to be attained quickly. After at least two weeks, the same monkeys will again be anesthetized and infused until the time that steady-state has been theoretically calculated to occur. At this time, arterial and venous blood will be collected, the animal will be euthanized, and the brain removed. Various brain regions will be dissected and samples assayed, by HPLC, to determine the levels of TP, serotonin (5-HT), a-MTP, and a-methylserotonin (a-M5HT); these data will allow determination of the differences in the distribution of these compounds.

Determination of the enzyme kinetic differences

Samples of the dissected brain regions from monkeys will be immediately assayed in an *in vitro* system for TP hydroxylase enzyme activity using both a-MTP and TP as substrates; this will allow determination of the kinetic differences towards the rate-limiting enzyme.

Major Findings:

Initial studies indicate that infusion of a-MTP for two hours does not alter TP or a-MTP plasma protein binding. a-MTP concentrations in various brain regions mirrors TP concentrations but are much lower. A chiral synthesis procedure has been developed; purification by HPLC is under investigation.

Significance to Biomedical Research and the Program of the Institute:

It has been reported that low levels of the serotonin metabolite 5-hydroxyindoleacetic acid in the cerebral spinal fluid are correlated with increased aggressive/impulsive behavior of patients. Determining the kinetic and distribution differences between TP and its tracer, a-MTP, will allow for the

quantitative determination of the neuroanatomical distribution of serotonin activity in patients with addictive and aggressive/impulsive behavior.

Proposed Course:

Finish assaying monkey tissues and plasma samples. Data analysis.

Publications:

None.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00292-04 LCS

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanisms of Altered Drug Metabolism Following Withdrawal from Ethanol

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. Shoaf	Senior Staff Fellow	LCS, NIAAA
Others:	C. Niebylski	NRC Fellow	LMBB, NIAAA
	B. Roberts	Visiting Fellow	LCS, NIAAA
	B. Song	Section Chief	LNG, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Unit of Pharmacokinetic Studies

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

2.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It is widely known that ethanol (ETOH) modulates the activity of the mixed function mono-oxygenase (MFO) system. How this drug metabolizing system changes upon ETOH withdrawal is unclear; thus, it was the purpose of our study to characterize possible changes by investigating activities of individual MFO isozymes. Enzymatic and immunochemical analyses revealed an ethanol inducible isozyme, CYP2E1, is rapidly degraded following withdrawal in several tissues, including brain, liver, kidney, and intestine. This suggests that any residual enhancement of ETOH clearance after ETOH withdrawal cannot be due to an increase in CYP2E1 activity. In contrast, other ETOH inducible isoforms of the MFO system remained transiently elevated following withdrawal, suggesting their mechanism of regulation differs from that of CYP2E1.

Project Description:Investigators:

S. Shoaf	Senior Staff Fellow	LCS, NIAAA
C. Niebylski	NRC Fellow	LMBB, NIAAA
B. Roberts	Visiting Fellow	LCS, NIAAA
B. Song	Section Chief	LNG, NIAAA

Objectives:

To determine the mechanisms responsible for the altered metabolism of therapeutic agents following ethanol (ETOH) withdrawal. Animal studies will be used to guide the development of human research protocols.

Initial studies will measure cytochrome P450 levels and activities, *in vitro*, and membrane lipid composition and fluidity in the rat following chronic ETOH exposure and at various times following the withdrawal of ETOH. Although the physiological changes that occur during the development of dependence are well characterized, there are few if any studies that have examined P450 activity or changes in membrane lipid composition during and after acute withdrawal.

Methods Employed:

Rats will be administered ETOH as part of a liquid diet; the Lieber-DeCarli diet, 36% of calories as ETOH, will be given for 21 days. Exposed and control animals will be pair-fed with the control diet isocalorically supplemented with dextrin-maltose. Microsomal preparations will be made from rat tissues. Rats will be killed immediately after the last feed period and at various times following ETOH withdrawal.

The activity of various cytochrome P450 isozymes will be measured *in vitro* using microsomal enzyme assays. By following the formation rate of a metabolite that is formed via the action of a single cytochrome P450 isozyme, the activity of that isozyme can be characterized. For example, acetanilide hydroxylation is mediated by the CYP2A2 isozyme. The demethylation of nitrosodimethylamine at low concentrations is mediated by CYP2E1 (the cytochrome P450 induced by ETOH). CYP2E1 also largely mediates the hydroxylation of p-nitrophenol (>90%). Antibodies will be used to immunoquantitate the amount of specific P450 isozymes. Western blots with computerized densitometry, as well as immunoprecipitation and radioimmunoassay, may be utilized. Changes in membrane composition will be determined by extracting lipids and analyzing the fatty acid distribution using gas chromatography with flameionization detection. The fluidity of microsomal preparations will be determined by incubating microsomes with lipophilic, fluorescent probes such as 1,6-diphenyl-1,3,5hexatriene (DPH). The fluorescence depolarization of the probe will be measured using both steady-state and time resolved fluorescence anisotropy with a Photon Technologies International (Princeton) LS-100 spectrofluorimeter.

Major Findings:

CYP2E1 is induced four-five fold by administration of the Lieber-DeCarli diet for three weeks. This induction was observed in several tissues, including brain, liver, kidney and intestine. After 12 hours of ETOH withdrawal, CYP2E1 levels returned to control, suggesting that the degradation of CYP2E1 is rapid and highly sensitive to tissue ETOH concentrations. Hepatic ETOH concentrations were undetectable 12 hours following ETOH withdrawal. Collectively, these data support the concept that ETOH "stabilizes" CYP2E1 and that this effect rapidly diminishes after ETOH is withdrawn.

Investigations of other P450 isoforms, including CYP3A (steroid inducible), CYP2B1, CYP1A1, and CYP2D6, indicate the first three are all inducible by ETOH,

with no change observed in the latter. All of these induced isoforms remained elevated at 24 hour withdrawal, suggesting their mechanism of regulation differs to that of CYP2E1. CYP2D6, an isoform implicated in the metabolism of several tricyclic anti-depressants, was not significantly altered during ETOH administration or withdrawal.

#### Significance to Biomedical Research and the Program of the Institute:

It has been reported that the efficacy of some psychotropic agents is lower in abstinent alcoholics compared to controls. It has been proposed that therapeutic concentrations are not obtained in abstinent alcoholics when they are dosed at levels normally given controls. The mechanism responsible for the lowered steady-state concentrations is proposed to be an increased rate of metabolism. It is important to understand the physical mechanisms underlying altered drug metabolism so that appropriate adjustments can be made in the dosage regimens of therapeutic compounds used for the treatment of abstinent alcoholics.

#### Proposed Course:

Having established a rapid pattern of CYP2E1 degradation in rats following ETOH withdrawal, it is of critical importance to reproduce these findings in human subjects. This may be achieved with the CYP2E1 specific probe chlorzoxazone. Using this approach, it will also be possible to evaluate the role of CYP2E1 induction in the clearance of ETOH during repeated exposure to alcohol. Molecular and rodent studies will also be pursued in an attempt to further delineate the effects of ETOH exposure on the MFO.

#### Publications:

None.





**LABORATORY OF MEMBRANE BIOCHEMISTRY AND BIOPHYSICS**



Annual Report of the  
Laboratory of Membrane Biochemistry and Biophysics  
Division of Intramural Clinical and Biological Research  
National Institute on Alcohol Abuse and Alcoholism  
October 1, 1993 to September 30, 1994  
Norman Salem, Jr., Chief

## Introduction

Although it has now been three years since the Laboratory of Membrane Biochemistry and Biophysics (LMBB) was created, this was the first year that it began to reach Laboratory status. The Sections of Nuclear Magnetic Resonance (NMR) and Mass Spectrometry have reached their full staffing levels, and the Section of Fluorescence now has its principal staff on board with the recruitment of Drs. Burton Litman and Drake Mitchell. Unfortunately, the difficulties created by the last minute denial of the NIAAA laboratory space in Building 49 have persisted and will persist into the next reporting period. In spite of the Laboratory being spread over five buildings and being forced to repeatedly move due to renovations, the Laboratory has nonetheless begun to foster the type of unique interactions between Sections and across disciplines that was envisioned in its creation. A variety of projects has been initiated, many of which are expected to provide important new results regarding the metabolism and membrane functions of essential fatty acids. The LMBB has begun to seed many collaborations within the Intramural Program as well as with our peers at Universities in the U.S. and abroad. The Laboratory will endeavor to be one of the recognized world centers for the advanced study of essential fatty acid, lipid, and membrane function.

## Office of the Chief

Major advances have been made with respect to the understanding of the relationship of alcohol abuse and essential fatty acids as well as in the development of animal models for their study.

The Laboratory has now completed the first arm of the first clinical study concerning *in vivo* essential fatty acid metabolism. The *in vivo* method takes advantage of non-toxic, stable isotope labeled fatty acids such as linoleic (18:2n6) and linolenic (18:3n3) acids given orally and deuterium labeled metabolites analyzed in plasma by GC/MS/NCI. This approach has now been used to show that humans are capable of elongation, desaturation, and export of both n-3 and n-6 fatty acids in the liver as metabolic end products such as 20:4n6, 22:5n6, and 22:6n3 incorporate deuterium from their 18-carbon precursors.

As we observed in studies of cats and rhesus macaques, the addition of long chain essential fatty acids, as in a high fish diet, leads to a decrease in the levels of deuterium labeled products such as 20:4n6. This was correlated with a decrease in plasma 20:4n6 after three weeks of the fish based diet. In the beef based diet, normal volunteer levels of net deuterium label in long chain polyunsaturates were the greatest, consistent with this diet supplying the lowest level of these lipids as preformed nutrients. Substantial progress has been made in assessing the variable of smoking in a second group of normal volunteers since this is the most appropriate control group for alcoholics. Initial results indicate a decrease in plasma arachidonic acid (20:4n6) in smokers that is observed in all three dietary groups. This study has laid the groundwork for the testing of *in vivo* metabolism in alcoholics which will begin in the next reporting period.

In addition, a study of premature and term infants has begun and the first phase of this work nearly completed. Qualitative features of infant essential fatty acid metabolism as related to postconceptional age are being examined. Our initial results indicate that most infants are capable of 20:4n6 and 22:6n3 biosynthesis from their 18-carbon precursors. These data are of critical importance for the development of optimal compositions of infant formula.

Progress has been made in developing a model of alcoholism that combines a "barely adequate diet" with respect to polyunsaturate status with a chronic alcohol challenge. Rhesus macaques and domestic cats are fed an olive oil or 1 wt % corn oil diet as the fat source and in some cases, antioxidant vitamins are lowered to remove the "safety factors". It is hypothesized that a diet low, but not inadequate, in 18-carbon essential fatty acids leads to a stressed system that is not able to withstand a repeated alcohol challenge to its acyl composition. We have recently demonstrated that cats given oral doses of ethanol for six months on such a diet have significant decreases in 22:6n3 in their brains and retinas and a compensatory increase in 22:5n6. The alcohol-exposed nervous system thus resembles that of the n-3 deficiency syndrome seen in early neural development when 22:6n3 supply to the fetus or newborn is inadequate. Their livers are also depleted of many long chain essential fatty acids. These losses in the structural lipids of neural membranes may underlie some of the functional losses in brain and retinal function that are associated with alcoholism.

Recent metabolic data have indicated an increased level of deuterium labeling in 22:5n6 and 22:6n3 in feline brain after alcohol exposure. This is accompanied by a four fold rise in aldehydes that are catabolic products of n-6 polyunsaturate peroxidation. These data suggest that there is an increased level of long chain polyunsaturate biosynthesis that attempts to compensate for a higher level of peroxidation caused by the prooxidative effects of alcohol.

Progress has been made in developing rapid methods of n-3 deficiency induction in animals *in vivo* as well as in cell culture. The artificial rearing method is used together with purified diets with defined fatty acyl compositions so that, for example, n-3 fatty acids may be removed from the artificial milk given to rat pups. A significant decline in brain 22:6n3 can be achieved in as little as seven days of early development under these diet limited conditions. Recent data have indicated that very high proportions of 18:3n3 relative to 18:2n6 may provide for the support of neural 22:6n3 levels in rats during early development, but ratios supplied by commercial milk formulas cannot. It is hypothesized that in women who abuse alcohol during pregnancy, nutritive factors related to maintaining 22:6n3 status in the infant or fetal brain may be crucial determinants of the fetal alcohol syndrome.

#### Section of Mass Spectrometry

It has been proposed that an important mechanism underlying many of the effects of ethanol is its capacity to alter polyunsaturate metabolism. In order to elucidate metabolic functions of polyunsaturated fatty acids and phospholipids in nervous tissues, alterations in processes involving incorporation, release, and remodeling of long chain polyunsaturates, which are especially enriched in brain, was investigated in the Section of Mass Spectrometry. We found that active incorporation of both 20:4n6 and 22:6n3 into rat brain, as well as enrichment of 22:6n3 in comparison to 20:4n6, occurred during the first month of life, indicating the importance of the nutritional supply at this early developmental stage for the accretion of polyunsaturates in the nervous system. One of the effects of ethanol on polyunsaturate fatty acid metabolism appeared to be the decrease in polyunsaturated fatty acid turnover in neuronal membranes as our results indicated the inhibition of polyunsaturated fatty acid incorporation and synaptosomal PLA<sub>2</sub> activity during activation. The elevated basal free fatty acid level in brain by chronic ethanol exposure may affect brain functions. The different hydrolysis profiles observed in synaptosomes and C-6 glioma cells may indicate the distinctive role of polyunsaturated fatty acid release in these systems. Receptor-mediated polyunsaturated fatty acid release as observed in glioma cells may be modified by ethanol. Due to the easily modifiable polyunsaturate profile as well as active lipid metabolism in C-6 glioma cells, this cell line may provide a valuable model to investigate the effect of ethanol and polyunsaturates on phospholipid remodeling processes in neuronal systems.

## Section of Fluorescence

The integration of intercellular signals is largely mediated by receptors in the plasma membrane of target cells. The role of polyunsaturated phospholipids in modulating G protein signaling and the mechanism of action of ethanol and general anesthetics in these systems are currently being investigated. In studies of the visual transduction system, ethanol was found to promote the formation of the G protein activating form of rhodopsin, MII, to a greater degree in a 16:0, 22:6n3 PC bilayer than in a 16:0, 18:1, PC bilayer. The greater efficacy of ethanol in membranes containing 22:6n3 acyl chains indicates that the action of ethanol is via a phospholipid-mediated mechanism. This is the first direct observation of such an effect of ethanol on a membrane receptor and is in distinct contrast to the apparent effect of ethanol on ligand gated channels, in which case a direct binding of ethanol to the channel is the favored mechanism. The enhanced meta II formation produced by acute ethanol exposure would result in hyperactivity of this pathway. The loss of unsaturated acyl chains in membrane phospholipids observed previously in this Laboratory as a result of chronic exposure to ethanol would be expected to diminish the effect of ethanol on the G protein signaling pathways. These combined effects may contribute to the tolerance to ethanol developed during chronic ethanol exposure. In other experiments, conditions were explored which better simulate the intracellular cytosol, where the activity of water is substantially lower than in the usual dilute buffer solutions used in *in vitro* experiments. The addition of neutral salts, which reduce water activity, was seen to increase the potency of ethanol. This observation may explain why in many *in vitro* experiments, high ethanol concentrations, relative to physiological ethanol levels, are needed to observe changes in system properties. The effect of water activity has generally been neglected but clearly must be taken into account when investigating cellular processes *in vitro*.

## Section of Nuclear Magnetic Resonance

Research performed in the NMR Section of LMBS highlights the importance of the lipid-water interface for membrane organization. The NMR investigations of alcohol-lipid interaction resolved the long-standing controversy about the primary point of action of ethanol on lipid bilayers. Because of the polar properties of ethanol, a location at the hydrophobic/hydrophilic interface has been favored by some researchers. However, such a location appears to contradict the well known fluidizing influence of ethanol on lipid hydrocarbon chains. The results obtained by the NMR Section show convincingly that ethanol binds at the interface. Ethanol binding results in an increase of the area per molecule, thus providing more volume in which the lipid acyl chains may move.

Further, results obtained on a series of phosphatidylcholines with increasing degree of fatty acid unsaturation show that unsaturation increases the area per molecule at the lipid-water interface. While saturated lipids show a rapid increase of the area per molecule with increasing temperature, the polyunsaturated lipids are less sensitive to temperature changes. Both observations are critical for the interaction of proteins with the lipid matrix as demonstrated by the interaction of a model peptide derived from the amino acid sequence of the envelope glycoprotein of HIV-I, and by protein kinase C with lipid membranes. The NMR Section has achieved a high standard of solid state NMR investigations using deuterium labeled compounds. The results provide a good foundation for investigations on complex mixtures of polyunsaturated lipids and lipid-protein interactions.





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Laboratory of Membrane Biochemistry and Biophysics  
October 1, 1993 to September 30, 1994

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00285-05 LMBB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Physiological Functions of Lipoyxygenase Products

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Karanian Research Physiologist LMBB, NIAAA

Others: N. Salem, Jr. Chief LMBB, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Membrane Biochemistry and Biophysics

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0.0

CHECK APPROPRIATE BOXES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mammalian platelets are high in lipoyxygenase activity and capable of enzymatically producing a number of n-6 and n-3 hydroxy fatty acids. Sub-micromolar levels of the 14-OH 22-carbon n-3 hydroxy fatty acids have been reported to specifically antagonize both the contractile effects of thromboxane both in vitro and in vivo, and its platelet aggregating effect. The 11-OH 22-carbon n-3 positional isomers were also potent in this regard. We have proposed that these hydroxy fatty acids specifically interact with the thromboxane receptor in both platelet and smooth muscle. Analysis of thromboxane binding parameters has shown a marked decrease in the affinity of the receptor for thromboxane occurred with a more mild decrease in the number of receptor sites. Inhibition of thromboxane binding correlates directly with inhibition of thromboxane-induced platelet aggregation by these hydroxylated derivatives. Since the 14-hydroxy derivatives are more active than the 11-OH-22 carbon n-3 positional isomers in the smooth muscle system as well as in platelet aggregation studies, the position of this moiety and the first double bond (n-3 vs. n-6) appears crucial; also, the S conformer was more active than the R form. Cycling of ethanol exposure by an inhalation method resulted in significant model improvements compared to the continuous ethanol exposure paradigm; weight gain was comparable to the controls with a zero attrition rate in the ethanol-dependent animals. Changes in liver fatty acyl content were similar to previous reports in continuously exposed rats. The fatty acyl content of the parent fatty acids in platelet and smooth muscle obtained from ethanol-exposed animals decreased in a time-dependent manner. Production of the hydroxylated derivatives from exogenous substrate was depressed in tissue obtained from long-term ethanol-exposed rats. However, production of the n-3 hydroxy fatty acids was elevated following short-term ethanol exposure (less than 14 days). Platelets obtained from the ethanol-treated rats were less responsive to thromboxane and produced less thromboxane. The observed changes in function correlated directly with decreases in thromboxane binding.

Project Description:Investigators:

J. Karanian	Research Physiologist	LMBB, NIAAA
I. Horvath	Visiting Fellow	LMBB, NIAAA
J. Poling	IRTA Fellow	LMBB, NIAAA
N. Salem, Jr.	Chief	LMBB, NIAAA

Objectives:

The major objectives of this project are to: (1) characterize the physiological functions of lipoxxygenase products of n-3 and n-6 polyunsaturated fatty acids; and (2) study the modification by ethanol of the function of these metabolites in platelet and vascular tissue.

Methods Employed:Synthesis of Hydroxylated Fatty Acids

Washed human platelets were prepared in a Tris-EDTA buffer and incubated for 30 minutes at 37°C with 1-200  $\mu$ M 22:5n6, 22:5n-5, 22:5n3, and 22:6n3. In some cases, rats were exposed to ethanol by an inhalation method for 1, 7, 14, 28, or 56 days. The effects of ethanol on the yield of the hydroxylated products and thromboxane were studied following the addition of the precursor fatty acids to the platelet suspension, *in vitro*. The positional isomers were separated and quantified using an HP-1090 HPLC equipped with a diode array UV detector (235 nm). The thromboxane levels (TXB2) were measured by RIA.

Extraction and Analysis of Fatty Acids

Lipids were extracted from the platelet and aortic tissue preparations using the Bligh and Dyer method. Fatty acids were transmethylated according to Morrison and Smith and analyzed by capillary gas chromatography.

Bioassays

Rats were used as a source of aortic rings and whole blood was obtained from the rat and human. Human and rat platelet aggregation was evaluated using a Chronolog Whole Blood Impedance Aggregometer. The effect of these hydroxylated fatty acids on cerebral blood flow (CBF) and thromboxane-induced changes in CBF of the rat was determined according to the method of Kety and Schmidt.

Binding Studies

Platelet suspensions ( $4-8 \times 10^8$  platelets/ml) were incubated with [ $^3$ H]-U46619 (5 nM) either alone or with 1000 fold excess unlabeled U46619 (800  $\mu$ l). For saturation binding studies, the platelet suspensions were incubated with 10 nM [ $^3$ H]-U46619 either alone or with increasing concentrations of unlabeled U46619 (50-750 nM). Specific binding was defined as total binding minus binding activity that could not be competed for by 10  $\mu$ M unlabeled U46619.

Inhalation Studies

The inhalation system was recharacterized utilizing a 12 hour cycle with regards to ethanol vapor exposure. The ethanol vapor concentration and blood ethanol level over a 24 hour period, animal body weight, attrition, withdrawal symptoms, and liver fatty acid content were assessed in comparison to continuous ethanol exposure. Rats were placed in inhalation chambers for 1, 7, 14, 28, or 56 days either in the presence or absence of ethanol vapors. Trunkal blood was collected in ACD (5:1) and washed platelet preparations were incubated with various precursor fatty acids. Following extraction the hydroxy fatty acids were quantified using a RP-HPLC system. In some cases whole blood was collected in citrate (3.8%) for assessment of fatty acyl content, platelet aggregation, platelet metabolite production and binding, and aortic rings were prepared for analysis of fatty acyl content.

Major Findings:

As previously reported, relatively low levels of the OH-22:6s specifically antagonize both the contractile effects of a thromboxane mimetic, U46619, and its platelet aggregating effect. These functional changes may be attributed to antagonism at the vascular smooth muscle cell or platelet thromboxane receptor. A marked decrease in thromboxane receptor affinity occurred (46%) with a more mild decrease (23%) in the number of receptor sites. Moreover, these findings correlate with changes in platelet function. In the concentration range evaluated (0.1-50  $\mu\text{M}$  14-OH-22:6n3), there is a direct correlation between inhibition of thromboxane receptor binding and inhibition of thromboxane-induced platelet aggregation.

In addition, human platelets were preincubated with 0.25  $\mu\text{M}$  of the 11-OH positional isomers of 22:5n3 and 22:6n3 and compared to previously tested hydroxy fatty acids in order to determine their effect on arachidonic acid-induced (0.25 mM) platelet aggregation. These hydroxylated derivatives reduced the slope of aggregation for arachidonic acid (AA). The rank order of all the fatty acids tested to date is 14-OH-22:6n3 (-68%) > 14-OH-22:5n3 (-43%) > 11-OH-22:6n3 (-32%) > 11-OH-22:5n3 (-30%) > 12-OH-20:4n6 (-21%) > 14-OH-22:5n6 (-14%)  $\geq$  12-OH-20:5n3 (-11%).

The 22-carbon n-3 hydroxylated fatty acids are effective antagonists *in vivo*. Thromboxane (est. 100 nM) was shown to decrease cerebral blood flow by more than 40% in the rat, whereas in the presence of 14-OH-22:6n3 (est. 250 nM), thromboxane did not induce a decrease in cerebral blood flow. The 20-C hydroxylated derivatives (12-OH-20:4n6 and 12-OH-20:5n3) were not significantly effective in this regard.

The 22-carbon hydroxylated metabolites of the n-3 parent fatty acids were the most potent inhibitors of TXA<sub>2</sub>-induced effects. These derivatives were at least one order of magnitude more potent in the platelet preparation (submicromolar) compared to the vascular smooth muscle preparations. Since the 14-hydroxy derivative is more active than the 11-OH-22 carbon n-3 positional isomers in the smooth muscle system as well as in the platelet aggregation studies, the position of this moiety and the first double bond (n-3 vs. n-6) appears crucial. In addition, the S-form of these derivatives has greater biological activity than the R-form.

Characterization of a Cycling Ethanol Exposure Model by an Inhalation Method

The effects of continuous ethanol exposure (24 hour/day) on mean or peak blood alcohol concentrations (BAC) in rat, body weight, attrition, withdrawal symptoms, and liver fatty acid content were compared to a cycling ethanol exposure (12 hr/day). The mean BAC was 187 mg% with a continuous ethanol vapor of 25 mg/L, and the peak BAC was 201 mg% with a cycling ethanol vapor of 34 mg/L. The weight difference was 18% between the continuously exposed animals and controls, whereas the weight difference (of 4% after 42 days) was not significant between cycling ethanol animals and controls. No animal attrition occurred with the cycling ethanol exposure, compared to a 16% attrition rate for the continuously exposed animals. All rats presented withdrawal symptoms.

Fatty acyl content, hydroxy fatty acid production in platelets (n-6 and n-3), thromboxane production and thromboxane binding, and platelet responses to thromboxane were evaluated *in vitro* following ethanol exposure *in vivo* by the cycling inhalation method for 1-56 days. The mean blood ethanol concentration was 87-196 mg% in all groups studied. The content of the parent fatty acids, 20:4n6 and 22:6n3, and their precursors (18:2n6 and 18:3n3), decreased in platelets and aortas obtained from ethanol-exposed animals. These changes generally correlated with a decreased capacity to produce cyclooxygenase and lipoxigenase products from the platelet. 12-OH-20:4n6 production from exogenous substrate (20  $\mu\text{M}$ ) was significantly decreased in platelets obtained from rats exposed to ethanol. The decrease was time-dependent and as great as 58%



following 56 days of ethanol exposure. Moreover, thromboxane production was reduced by at least 30% at all time points studied. Similarly, 14-OH-22:6n3 production was reduced by 25% and 56% following 28 and 56 days ethanol exposure, respectively. However, this 22-C n-3 derivative was elevated by 77%, 110%, and 118% following 1, 7, and 14 days ethanol exposure, respectively.

Platelet aggregation to thromboxane (arachidonic acid, 0.25 mM) was significantly reduced in a time-dependent manner as was previously reported for the contractile responses of similarly treated vascular tissue. The slope of aggregation decreased by 20%, 32%, 57%, 72%, and 88% following 1, 7, 14, 28, and 56 days of ethanol exposure, respectively. Changes in thromboxane receptor binding correlated significantly with the observed decrease in thromboxane-induced aggregation ( $r=0.66$ ,  $p<0.01$ ). The percent of control binding was 91, 82, 77, 37, and 39 for  $^3\text{H}$ -U46619 following 1, 7, 14, 28, and 56 days of ethanol exposure, respectively.

#### Significance to Biomedical Research and the Program of the Institute:

The biological properties of additional n-3 and n-6 hydroxy fatty acids have been characterized and compared to the 22:6n-3 hydroxy fatty acids. The 22 carbon n-3 hydroxy fatty acids are the most potent biologically with regards to their inhibition of thromboxane-induced platelet aggregation and vascular contraction. These properties may be directly related to their degree of structural analogy with thromboxane which may determine their ability to decrease thromboxane receptor affinity. Short-term ethanol exposure increases the production of these n-3 hydroxy fatty acids and correlates with a decrease in platelet and vascular smooth muscle function, whereas long-term ethanol exposure depletes both the content of the parent fatty acids and the capacity to produce these n-3 metabolites in platelet. Moreover, ethanol-induced decreases in platelet thromboxane production and receptor binding correlate with a dramatic and time-dependent decrease in thromboxane-induced platelet aggregation. We have previously reported that enrichment of the n-3 fatty acids in the diet countered the fatty acid depleting effects of ethanol in platelets. Therefore, lipid remodeling by nutritional means of the fatty acyl content and hydroxy fatty acid profile in platelet may eventually offer clinical applications in the control of thromboxane-mediated responses related to thrombosis and homeostasis.

#### Proposed Course:

The production and biological activities of the n-6 and n-3 hydroperoxy fatty acids will be biosynthesized, characterized, and compared to the effects of the hydroxylated derivatives in platelet and vascular smooth muscle. Emphasis will be placed on the ability of these lipoxygenase products to alter cell function and the mechanism by which this may be accomplished. The effects of the positional isomers on eicosanoid metabolism and receptor binding parameters will be determined. The various hydroperoxy and hydroxy metabolites will be screened for their effects on the electrophysiological properties of cortical cells and pinealocytes. The proposed essentiality of the 22 carbon n-3 lipoxygenase products will be addressed in this way.

#### Publications:

Karanian JW, Kim HY, Salem N Jr. Lipoxygenase stimulating effects of hydroxylated docosahexaenoates, Prostaglandins, Leukotrienes, Essential Fatty Acids 1994;50:271-8.

Karanian JW, Kim HY, Shingu T, Salem N Jr. Inhibitory effects of n-6 and n-3 hydroxy fatty acids on thromboxane (U46619)-induced smooth muscle contraction, J Pharmacol Exp Ther 1994;270(2):1-5.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 AA 00262-10 LMBB

## PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Desaturation of Essential Fatty Acids Using Stable Isotope/Mass Spectrometry

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. Pawlosky Senior Staff Fellow LMBB, NIAAA

Others: N. Salem, Jr. Chief LMBB, NIAAA  
G. Ward Visiting Fellow LMBB, NIAAA

## COOPERATING UNITS (if any)

None.

## LAB/BRANCH

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## SECTION

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## INSTITUTE AND LOCATION

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## TOTAL STAFF YEARS:

1.4

## PROFESSIONAL:

1.4

## OTHER:

0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The essential fatty acids (EFA), linoleate (18:2n6) and linolenate (18:3n3), are desaturated and elongated to arachidonate (20:4n6) and docosahexaenoate (22:6n3), respectively. A stable isotope gas chromatography-mass spectrometry (GC-MS) method is being used to examine the effects of ethanol on the production of polyunsaturated fatty acids in vivo. Ethanol consumption has been shown to lower the levels of 20:4n6 and 22:6n3 in the plasma and liver of several mammalian species. Alcohol, in combination with a diet having low amounts of EFAs, has been shown to remodel long chain polyunsaturated fatty acids in the nervous system in felines, whereby 22:5n6 replaced 22:6n3 in the brain and retina. In felines, alcohol-exposed animals had higher levels of deuterium-labeled 22:5n6 and 22:6n3 in their brains compared to controls. As a result of alcohol exposure, the level of the 4-hydroxy-nonenal, a product of n-6 fatty acid peroxidation, increased by four fold in the brains of felines. This suggested that increased synthesis of the deuterium-labeled 22:5n6 and 22:6n3, which accompanied higher levels of lipid peroxidation, may compensate for the losses of long chain polyunsaturated fatty acids. In related work carried out in rhesus macaques, alcohol exposure led to a decline in the level of neural 22:6n3 and an increase in the level of 22:5n6. A greater 22:5n6/22:6n3 ratio was also reflected in the plasma of the alcohol-exposed animals.

Project Description:Investigators:

R. Pawlosky	Senior Staff Fellow	LMBB, NIAAA
N. Salem, Jr.	Chief	LMBB, NIAAA
G. Ward	Visiting Fellow	LMBB, NIAAA

Objectives:

The major objectives of this project are to: (1) examine dietary factors which may regulate desaturation/elongation of essential fatty acids (EFA) *in vivo* in mammals; (2) analyze the role of ethanol in altering the formation and catabolism of 20- and 22-carbon polyunsaturated fatty acids; and (3) investigate the relationship between ethanol exposure and lipid peroxidation.

Methods Employed:Feline StudiesAdult Feline Alcohol Study

Seven adult male cats were acclimated for two months to a purified diet containing 10% fat (9:1 hydrogenated coconut oil:corn oil) with no 20- or 22-carbon polyunsaturates. The other macro nutrients consisted of rice flour and casein and the vitamin and mineral contents were provided at adequate levels for this species. Desaturation/elongation activities were determined by the appearance of the deuterium-labeled products in the blood, liver, and brain following oral doses of deuterium-labeled 18:2n6 and 18:3n3 ethyl esters given as 10 mg doses each day for 10 days prior to euthanasia. Each day for six months, four of the cats were given oral doses of ethanol, 1.2 g/kg (in gelatin capsules containing a 95% alcohol solution). The plasma and tissue fatty acyl compositions were obtained using gas chromatography. The animals were euthanized with an overdose of sodium pentobarbital and the organs were excised. The deuterium-labeled metabolites and the lipid composition of the brains, liver, and retinas were analyzed using gas chromatography and GC-MS.

Primate Alcohol and Diet Study

This is the third year of a study investigating the consequences of long-term alcohol exposure on essential fatty acid metabolism in rhesus macaques. The objectives are to determine the effects that a low essential fat diet with lowered levels of vitamins E and C (40 IU/kg and 250 mg/g) and alcohol exposure has on polyunsaturated fatty acid metabolism, the development of liver pathology, and brain lipid composition. A long-term goal is to evaluate whether a diet containing long chain polyunsaturated fatty acids will attenuate some of the alcohol-induced liver pathology. Animals are offered an artificially sweetened, 7% alcohol solution on an ad lib, self-select basis. Alcohol consumption is monitored by using a blood alcohol level enzyme assay and animal alcohol consumption records. Liver status is evaluated using clinical biochemistry blood panels and liver histopathology. Fatty acyl composition analyses were determined from biopsy samples of the liver and brain from controls and alcohol-exposed animals. In carrying out the brain biopsy, surgical craniotomy was used to remove a circular, 100 mm diameter piece of the pyramidal skull bone, then approximately 100 mg of tissue (a 3 x 100 mm core section) was excised from the right lobe of the cerebral cortex using a biopsy needle.

Analytical Chemistry Procedures

Tissue and plasma samples were extracted using the Bligh and Dyer total lipid extraction method. The fatty acids were derivatized to either their pentafluorobenzyl esters (PFB) or methyl esters using PFB bromide containing diisopropylethylamine in acetonitrile (1:10:1000) or BF3 in methanol (14% w/v). The PFB derivatizing reagent was evaporated under nitrogen and the residue dissolved in 1 ml hexane for GC/MS analysis. Fatty acid PFB esters were analyzed on a 0.25 mm x 30 m DB-FFAP capillary column using splitless injection with an

oven temperature program of 80°-185°@20°/min, 185°-240°@10°/min and held for 30 minutes. The PFB esters were detected as the M-PFB ion with selected ion monitoring using a Hewlett-Packard 5989 mass spectrometer operated in the negative chemical ionization mode. The fatty acid PFB esters were characterized by their elution time, the major fragment major ions, and by comparison to authentic fatty acid standards. The deuterium-labeled products were quantified using the relative response factors of the individual analytes compared to an internal standard, 23:0. Fatty acid methyl esters were analyzed by GC-FID using a split injection with a 10:1 ratio on a 0.25 mm x 30 m DB-FFAP capillary column using split injection. The oven temperature was programmed from 140°-180°@4°/min-210°@1°/min-245°/30° min and held for 19 minutes. Fatty acids were quantified from a known amount of an internal standard, 23:0.

The 4-hydroxy-alkenals were analyzed as their pentafluorobenzyl oxime trimethylsilyl (O-PFB, TMS) derivatives with selected ion monitoring using a negative chemical ionization GC-MS technique. Tissue samples (3-50 mg) were homogenized in a mixture of 400 ul of methanol containing 50 ug/ml BHT, 200 ul of a 2 mM EDTA buffer, pH 7.0, and 200 ul of 0.1M PIPES buffer containing 50 mM PFBHAHCl, pH 6.5. Samples were incubated at room temperature for five minutes, extracted with hexane, and the hexane was evaporated under nitrogen. The samples were dissolved in 50 uL of a 1:1 mixture of BSTFA:TMSI and incubated at 80° for five minutes. Known amounts of the deuterium-labeled 4-hydroxy-nonanal and 4-hydroxy-hexenal were added to the samples prior to derivatization to quantify the analytes. Samples were analyzed on a DB-5 column, 0.25 mm x 30 m with an oven temperature program of 80°-240°@10°/min. The 4-hydroxy-alkenals eluted concurrently with the deuterium-labeled standards.

#### Major Findings:

##### Feline Alcohol Study

The amount of deuterium incorporation into the long chain polyunsaturated fatty acids was used to determine their relative production. In the brains of alcohol-exposed animals, the levels of deuterium-labeled 22:5n6 was ten fold higher and the level of deuterium-labeled 22:6n3 was three fold higher compared to the controls. The level of the deuterium-labeled 22:4n6 was about three fold higher in these same animals and there was no difference in the level of deuterium-labeled 20:4n6. Previous analyses of the fatty acyl composition of the total lipid extract of these brains showed that the level of 22:6n3 had decreased by 20% and the level of 22:5n6 increased in the alcohol-exposed animals. In the acidic phospholipid fraction (phosphatidylinositol and phosphatidylserine), the level of 22:6n3 decreased by 26% and the level of 22:5n6 increased by 30% in the alcohol-exposed animals. GC-MS analyses indicated that the level of 4-hydroxy-nonanal had increased approximately four fold in the brains of the alcohol-exposed animals compared to the controls. It appeared from these data that synthesis of long chain polyunsaturated fatty acids in the brains was not inhibited by alcohol but rather stimulated. This appeared to be accompanied by increased EFA catabolism ascribed to the prooxidant effects of ethanol. Similarly, it was found that when mice were exposed to ethanol for two weeks, the level of 4-hydroxy-nonanal increased between four and five fold in the brain compared to control animals.

##### Primate Alcohol Study

Animals maintained on the low EFA diet plus alcohol underwent two liver and one brain biopsy this year. The pathologist's findings from the liver sections indicated that animals receiving alcohol had moderate levels of steatosis with inflammation compared to the controls. The livers of the alcohol-exposed animals were infiltrated with rare neutrophils and leukocytes and contained moderate amounts of fat dispersed diffusely throughout the liver. Fatty acyl analyses of the plasma revealed the alcohol-exposed animals had lower levels of both 20:4n6 and 22:6n3 compared to controls. The level of 22:6n3 in the plasma had decreased by as much as 70% compared to controls. In the brain, 20:4n6 levels were about the same for both control and alcohol-exposed animals. However, in the brain of



the alcohol-exposed animals, the level of 22:6n3 was on average 21% lower than in the brains of chow fed animals of approximately the same age. The level of 22:5n6 was 200% higher in the alcohol-exposed animals compared to the same controls.

#### Significance to Biomedical Research and to the Program of the Institute:

The development of a noninvasive and highly sensitive method for the determination of essential fatty elongation, desaturation, and accretion based on stable isotope technology has been implemented for both human and animal studies. The latter are being used to determine the relative contributions of the amounts and types of fatty acids in the diet and alcohol exposure to EFA deficiencies. The alcohol-induced loss of tissue polyunsaturates, the subsequent decrease in eicosanoid production, and the increased levels of lipid peroxidation products may provide an explanation of some of the pathological consequences of alcohol abuse. In animal studies, it appears that alcohol in combination with a low essential fatty acid diet contributes to losses of specific long chain polyunsaturated fatty acids in the nervous system. Prolonged alcohol exposure in animals induces the loss of 22:6n3 in the CNS where it is maintained in high concentration. This suggests that there may be a need to supplement the diet of alcoholics with long chain n-3 fatty acids.

#### Proposed Course:

Investigations into the long-term effects of alcohol on essential fatty acid status, the development of alcohol-induced liver pathology, and CNS lipid composition changes in rhesus macaques are being continued. A second brain biopsy for all animals is planned for this year in order to compare the effects of a low EFA diet alone to the combination of a low EFA diet and alcohol. A stable isotope study using deuterium-labeled 18:2n6 and 18:3n3 in rhesus macaques is in progress. It will evaluate the effect of alcohol on the uptake and conversion of essential fatty acids to the long chain polyunsaturated fatty acids and how these fatty acids are then partitioned into plasma lipoproteins and red blood cells.

#### Publications:

Pawlosky RJ, Barnes A, Salem N Jr. Essential fatty acid metabolism in the feline: The relationship between liver and brain production of long chain polyunsaturated fatty acids, *J Lipid Res*, in press.

Salem N Jr., Pawlosky, RJ. Arachidonate and docosaheptaenoate biosynthesis in various species and compartments *in vivo*, *World Rev Nutr Diet*, in press.

Salem N Jr., Pawlosky, RJ. *In vivo* studies of the supply of polyunsaturates to the brain in various mammals. In: Spector A, Pownall H, Gotto A, eds. *Proceedings of the American Heart Association Conference on Omega-3 Fatty Acids in Nutrition, Vascular Biology and Medicine*, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00235-12 LMBB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nutritional Effects on Essential Fatty Acid Composition

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: N. Salem, Jr. Chief LMBB, NIAAA

Others: Y. Denkins IRTA Fellow LMBB, NIAAA  
J. Hibbeln Clinical Associate LMBB, NIAAA  
R. Pawlosky Senior Staff Fellow LMBB, NIAAA  
M. Reyzer Chemist LMBB, NIAAA  
G. Ward Visiting Fellow LMBB, NIAAA  
B. Wegher Chemist LMBB, NIAAA

COOPERATING UNITS (if any)

USUHS, Pediatrics (J. Woods); INTA University of Chile, Pediatrics (R. Uauy)

LAB/BRANCH

Laboratory of Membrane Biochemistry and Biophysics

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

5.1

PROFESSIONAL:

3.1

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Two human trials were performed in order to directly assess the ability of human adults and infants to metabolize linoleic (18:2n6) and linolenic (18:3n3) acids to their metabolic "end products", arachidonic (20:4n6) and docosahexaenoic (22:6n3) acids. A method recently developed in this laboratory was used in which the deuterated precursors are given orally and the deuterated, elongated, and desaturated products analyzed by a highly sensitive and specific GC/MS method after their release into the plasma. Thus, a noninvasive method for the in vivo measurement of these liver enzymes can be performed. Our results indicate that humans do have the enzymatic capabilities to perform these interconversions and do perform them, in vivo. The biosynthesis of 20:4n6 has been demonstrated for the first time in human infants and adults. Our adult studies have suggested that the level of dietary intake of preformed long chain polyunsaturates is also a crucial regulatory factor for plasma polyunsaturate accretion. For example, a fish based diet that is high in 20:4n6, 20:5n3, and 22:6n3 leads to a decrease in the net synthesis of these fatty acids.

Project Description:Investigators:

N. Salem, Jr.	Chief	LMMB, NIAAA
Y. Denkins	IRTA Fellow	LMMB, NIAAA
J. Hibbeln	Clinical Associate	LMBB, NIAAA
R. Pawlosky	Senior Staff Fellow	LMBB, NIAAA
M. Reyzer	Chemist	LMMB, NIAAA
R. Uauy	Director and Professor	INTA U of Chile
G. Ward	Visiting Fellow	LMMB, NIAAA
B. Wegher	Chemist	LMBB, NIAAA
J. Woods	Neonatology Fellow	Dept. Pediatrics USUHS

Objectives:

The major objectives of this project are to: (1) investigate the functions of polyunsaturated fatty acids with particular reference to the nervous system; (2) determine whether docosahexaenoic acid is an essential nutrient for mammals; (3) test the hypothesis that ethanol exposure leads to alterations in essential fatty acid composition and, in particular, a loss of polyunsaturates from membrane phospholipids; (4) test the hypothesis that the combination of chronic ethanol consumption and a marginal diet with respect to polyunsaturate level maintenance may lead to adverse consequences for organ function, particularly the brain and liver; and (5) test the hypothesis that an alteration in the polyunsaturate composition of the diet with respect to polyunsaturate status may be used to reverse or prevent alcohol-induced losses in membrane polyunsaturates.

Methods Employed:Lipid Extraction

Lipids were extracted from brain, liver, heart, aorta, platelets, and plasma according to the method of Bligh and Dyer using chloroform-methanol-water in the ratios of 1:2:0.8. Erythrocytes were extracted using the method of Reed. A small amount (50 mg) of the antioxidant BHT was added to each sample. Tissues were homogenized using a Polytron (Brinkman) cavitation type instrument and then one volume of chloroform and water was added to partition the mixture into two phases. Lipid extracts were subjected to a solid phase extraction method developed in the lab when composition of lipid subclasses was desired. In some cases, phospholipid classes were separated by thin layer chromatography using the method of Rouser and coworkers; the solvent system employed was chloroform-acetone-methanol-acetic acid-water (5:2:1:1:0.5). The gel containing individual phospholipid classes was scraped off of the plates and directly transmethylated, as above.

Fatty Acid Analysis

Portions of these total lipid extracts were evaporated under a stream of nitrogen and transmethylated with 14 wt% BF<sub>3</sub> in methanol for two hours at 100°C using a modification of the method of Morrison and Smith. Hexane was used in place of benzene as a cosolvent in order to minimize exposure of lab personnel to the latter agent. It was observed in studies of fish oil, plasma, and brain samples that this substitution produced no observable losses in any of the component fatty acids. The methyl ester derivatives were extracted into hexane and evaporated under nitrogen for injection into an HP-5890 gas chromatograph equipped with a flame ionization detector. A 30 m x 0.25 mm ID fused silica capillary column (J & W Scientific) was used with hydrogen as carrier gas at a linear velocity of 50 cm/sec. Injector, detector, and initial column temperatures were 240°C, 240°C, and 130°C, respectively. The oven temperature rose at a rate of 4°C/min until 175°C and thereafter at a rate of 1°C/min until 210°C was reached. Finally, the oven temperature was increased at 30°C/min until 240°C was reached and the remainder of the run was at this temperature. Samples

were injected by means of a 7673A autosampler with a split ratio of 10:1. Fatty acids were identified by comparison to primary and secondary standards and by electron impact mass spectrometry on an HP-5970 mass selective detector.

#### Human Protocol

The *in vivo* metabolism of deuterated 18:2n6 and 18:3n3 was investigated in normal volunteers on their ad lib diets or in the third week of two controlled diets served on our ward. These diets consisted of a beef based or a fish based diet; the beef diet closely resembled the traditional American diet, whereas the fish diet was high in long chain polyunsaturates including 20:4n6, 20:5n3, and 22:6n3. Each subject was thus evaluated three times in a sequential fashion. Food records were kept for two weeks while patients were on their ad lib diet and the data analyzed using the Minnesota Data Base and other related instruments so that the nutrient content of the diet of each individual was calculated. Blood samples were withdrawn after 0, 8, 24, 72, 96, and 168 hours after a single oral dose of the two deuterated fatty acids. Plasma samples were prepared for fatty acyl compositional analysis and for GC/MS/NCI analysis of the amounts of each n-3 and n-6 deuterated metabolite of 18:3n3 and 18:2n6, respectively.

#### Artificial Rearing

When control of the fatty acyl composition of the diet during early development in the rat is required, animals are artificially reared on a purified diet. Four day old Sprague-Dawley or Long Evans rat pups are gastrostomized and raised until day 21 of life using the "pup in a cup" method. Milk formulas with varying levels and ratios of 18:2n6 and 18:3n3 and with or without long chain n-3 and n-6 polyunsaturates are pumped into their stomachs. One group was fed on an n-3 deficient diet and protein and other nutrient sources were carefully chosen and analyzed to ensure very low levels of fat and of n-3 fatty acids were present. Before weaning, the pups undergo sensorimotor development testing including such variables as righting from an inverted position, time of eye opening, development of fore- and hind limb grasping responses, and development of integrated locomotor coordination. After weaning, young rats are assessed on spatial, operant, and latent learning tasks. The spatial tasks will include the Morris water maze task and a spatial learning task involving habituation to olfactory cues in an open field. The operant tasks involve a step-wise development of bar pressing responses for food rewards, beginning with shaping of the bar pressing response and left-right and brightness discrimination and leading to more complex tasks such as delayed responding and matching to concurrent fixed and variable ratio schedules of reinforcement. Latent learning tasks involve the learning of food preferences through olfactory cues from conspecifics. Long chain polyunsaturates in brain and retina will be analyzed and correlated with indices of brain function as measured by these behavioral endpoints.

#### Major Findings:

A study of adult normal volunteer smokers and nonsmokers was performed in order to determine whether humans can elongate, desaturate, and export linoleic (18:2n6) and linolenic (18:3n3) acids and their metabolites. The regulation of this activity by various diets where the fatty acyl composition is altered has also been investigated using three diets including ad lib, beef based, and fish based given in that order; the latter two diets were given for three weeks with the assessment of *in vivo* metabolism during the third week. This initial study has formed the basis for the investigation of the *in vivo* fatty acid metabolism in alcoholics during withdrawal, most of which are smokers. This was the first controlled clinical study of the potentialities of humans in this respect.

Our results indicate that every individual studied (n=19) expresses the enzymatic activities of sequential essential fatty acid elongation and desaturation. In contrast to claims by Emken and coworkers, we have clearly demonstrated that arachidonic acid (20:4n6) is biosynthesized from 18:2n6 in humans *in vivo*. In the beef diet, the accumulation of plasma deuterated 20:4n6 and 22:6n3 was much higher than in the other two diets when analyzed either by integration of the



time course curves or when comparing the maximal value attained after two-four days. This was consistent with the lower intake of preformed long chain polyunsaturates in this diet. Conversely, the accumulation of deuterated long chain polyunsaturates in the same individuals while consuming the fish diet was the lowest, corresponding to the highest dietary intake of these nutrients. These data support the concept that the net biosynthesis (synthesis minus catabolism) of long chain polyunsaturates is depressed by their dietary intake, i.e., by a mechanism that has been termed "product inhibition".

A second human study has been initiated in this reporting period in collaboration with Dr. Ricardo Uauy of the Department of Pediatrics, INTA University of Chile, Santiago, Chile. In this study, the ability of human infants of various gestational ages and with varying essential fatty acids in their milk formulas are being investigated. Thus far, eight infants have been studied and all of them have shown a capacity to elongate and desaturate 18 carbon essential fatty acids. Most of them have been capable of processing these fatty acids to their usual metabolic end products, i.e., 18:2n6 to 20:4n6 and 18:3n3 to 22:6n3. One infant that had a suspected peroxisomal disorder was studied and had a relatively low capacity to accumulate deuterated plasma long chain polyunsaturates, consistent with the emerging view of the central involvement of a deficiency in these lipids, particularly 22:6n3, in the functional disturbances of the nervous system related to peroxisomal enzyme losses. These were the first studies that demonstrated that newborn infants have an active desaturating system, presumably localized in their livers.

A new model of n-3 deficiency is being developed in which the maternal transfer of n-3 fatty acids in the milk is prevented during the first three weeks of life by artificially rearing the pups on an artificial milk containing purified components with very low levels of 18:3n3 and no long chain n-3 fatty acids. Previous experiments have suggested that a significant decline in brain 22:6n3 can be achieved in 10 day old rats using this approach. This approach continues to be developed using a synthetic diet in which individual fatty acids can be deleted or supplemented. A reduction in mortality was observed when the protein source was changed to decrease casein and substitute whey or soybean protein.

Progress has been made in establishing behavioral indices of higher level brain functions in rodents as they are hypothesized to be compromised by n-3 fatty acid deficiency. More specifically, rats are given an open field task using at least two discreet olfactory stimuli together with salient cues in another modality. They are subsequently challenged with being placed in the opposite end of the apparatus and dishabituation measured when the olfactory cues are in the same or the opposite orientation to that given initially. The series of tasks attempts to determine whether the animals have constructed a cognitive map of the olfactory layout of their environment which is independent of their position. In this way, deficits in a number of behavioral properties can be assessed including activity, exploration, habituation, dishabituation, spatial learning, latent learning, memory consolidation, and cognitive mapping based on configural associations between two modalities.

A study was performed in which the 18:2n6 to 18:3n3 ratios in the formulas were varied from 10:1 to 1:20 and brain fatty acyl distributions evaluated after artificially rearing animals from day 5 to day 20 of life. The mean level of brain 22:6n3 increased as the amount of 18:3n3 was increased and was the same as dam-reared controls in the 1:20 group. This model provides an excellent and quick model of n-3 deficiency with which the consequences of the effects of alcohol on the nervous system can be evaluated. More generally, it allows for the evaluation of individual fatty acid nutrients in neural development and function.

A cell culture model of n-3 deficiency is also under development using a human retinoblastoma cell line. These cells have been successfully grown in fat free media and the polyunsaturates were substantially depleted in this way. Current

studies are attempting to supplement 18:2n6 which will be incorporated and metabolized to 22:5n6 by the cells in order to make the same substitution that is observed in the retina and brain after several generations of deficiency.

#### Significance to Biomedical Research and the Program of the Institute:

These studies have for the first time described essential fatty acid biosynthesis in humans. Both adult and infant synthesis of long chain polyunsaturates such as arachidonic and docosahexaenoic acids have been demonstrated; however, these data should not be interpreted to indicate that the longer chain n-3 and n-6 metabolites are unnecessary. These data have important implications for the analysis of which dietary fats are essential nutrients. These basic data will lay the foundation for the study of human pathology with respect to essential fatty acid metabolism.

#### Proposed Course:

Our *in vivo* studies of human essential fatty acid metabolic capacity will continue and several new variables will be evaluated. Alcoholics will be admitted to this protocol as well as normal volunteer smokers, their most appropriate control group. Our infant study will be extended to an evaluation of gestational age and dietary fatty acid regulation of fatty acid metabolism.

A second major line of investigation that is being actively pursued is to use a "barely adequate diet" in animals exposed to alcohol in order to better simulate what is believed to be the case in many alcoholics. This model attempts to lower essential fatty acids to levels that are considered to not induce any overt clinical symptomatology and to thus remove the safety factors. In some cases, antioxidant vitamins and minerals are also treated in the same way as they may also prevent some of the deleterious effects of alcohol abuse when their levels are elevated in the diet. This model will be extended to encompass neural development with an alcohol challenge in cats to simulate the fetal alcohol syndrome. In addition, collaborative projects with Fetal Alcohol Research centers will allow the analysis of cord blood and milk from human infants and their alcoholic mothers.

#### Publications:

Knapp H, Hullin F, Salem N Jr. Asymmetric incorporation of dietary n-3 fatty acids into membrane aminophospholipids of human erythrocytes, *J Lipid Res*, in press.

Salem N Jr. The biological role of polyunsaturated fatty acid peroxidation versus membrane functions in the nervous system. In: Spector A, Pownall H, Gotto A, eds. *Proceedings of the American Heart Association Conference on Omega-3 Fatty Acids in Nutrition, Vascular Biology and Medicine*, in press.

Salem N Jr, Pawlosky RJ. Health policy aspects of lipid nutrition and early development, *World Rev Nutr Diet*, in press.

Salem N Jr, Ward GR. Are omega-3 fatty acids essential nutrients for mammals?, *World Rev Nutr Diet* 1993;72:128-47.

Salem N Jr, Ward GR. The effects of ethanol on polyunsaturated fatty acid composition. In: Alling C, Sun G, eds. *Alcohol, Cell Membranes and Signal Transduction in Brain*. New York: Plenum Press, 1993;33-46.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00072-03 LMBB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Fluorescence Studies of Biophysical Properties of Polyunsaturated Phospholipids

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	B. Litman	Section Chief	LMBB, NIAAA
Others:	K. Hines	Biologist	LMBB, NIAAA
	D. Mitchell	Senior Staff Fellow	LMBB, NIAAA
	C. Niebylski	IRTA Fellow	LMBB, NIAAA
	N. Salem, Jr.	Chief	LMBB, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Membrane Biochemistry and Biophysics

SECTION

Section of Fluorescence Studies

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

2.35

PROFESSIONAL:

1.85

OTHER:

0.50

CHECK APPROPRIATE BOXES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Chronic alcohol exposure is known to cause resistance to ethanol-induced membrane disordering measured in vitro, usually referred to as tolerance. Initial studies on the phospholipid acyl chain dependence of the effects of alcohol demonstrate that polyunsaturated acyl chains potentiate the effect of alcohol. This finding, coupled with the observation made in this Laboratory, that alcohol depletes the long chain polyunsaturated fatty acids, such as 20:4n6 and 22:6n3, may provide an explanation for the development of the tolerance effect. A novel fluorescence parameter termed the fractional volume was derived from dynamic anisotropy measurements on DPH in lipid bilayers. This parameter was used to derive information about acyl chain packing properties in these systems. A comparison of this parameter in phospholipids of varying acyl chain unsaturation shows that one must elevate the temperature to 37 C in di-14:0 PC to attain equivalent values of the fractional volume attained at 30 C and 20 C in 16:0, 22:6 PC and di-20:4 PC, respectively. Thus, while saturated acyl chains have more intramolecular orientational degrees of freedom than polyunsaturated acyl chains, at physiological temperatures, polyunsaturated acyl chains yield a bilayer with lateral acyl chain packing properties more conducive to conformational changes of integral membrane proteins than do saturated acyl chains. The phase behavior of di-18:0 PC, di-22:6 PC, a mixture of these two lipids, and 18:0, 22:6 PC indicates that special domain features are present in the mixed acyl chain phospholipid systems that are not seen in the symmetrically substituted phospholipids; these structures may be related to the special physical properties these mixed chain lipids impart to bilayers.

Project Description:Investigators:

B. Litman	Section Chief	LMBB, NIAAA
K. Hines	Biologist	LMBB, NIAAA
D. Mitchell	Senior Staff Fellow	LMBB, NIAAA
C. Niebylski	IRTA Fellow	LMBB, NIAAA
N. Salem, Jr.	Chief	LMBB, NIAAA

Objectives:

The major objectives of this project are to: (1) study phospholipid acyl chain packing properties in order to determine what unique structural features, such as lateral domains, polyunsaturated phospholipids impart to biological membranes; (2) examine the effect of ethanol exposure on lateral and transmembrane lipid organization and to determine whether these effects are potentiated by polyunsaturated phospholipids; (3) evaluate the influence polyunsaturated lipids have on the function of biological membranes and membrane proteins; and (4) develop novel fluorescence methods of analysis in order to detect lipid domains in membranes.

Methods Employed:Purification of Cis-Unsaturated Fatty Acids

Separation of synthetic cis and trans isomers of docosapentaenoic methyl ester was accomplished using reverse phase HPLC. Docosapentaenoic acid methyl ester was purified on a C18 4 $\mu$ m, 3.9 x 300 mm column with a gradient from 80% acetonitrile/H<sub>2</sub>O to a final 100% acetonitrile at 1 ml/min. The purification is being scaled up to the preparative level to increase the efficiency of purification. A gram of purified acid was prepared and limited quantities of mixed acid phospholipids were synthesized containing 22:5n6 at the sn-2 position of PC, PE, and PS.

Differential Scanning Calorimetry of Membrane Preparations

Differential Scanning Calorimetry (DSC) was accomplished using a Mettler DSC-30 calorimeter which includes a nitrogen dewar/heater temperature controller for controlled cooling to -170°C. Samples were prepared by placing 1 to 10 mg of lipid in organic solvent in an aluminum sample crucible and drying under nitrogen. An excess of water (5  $\mu$ l) was then added and the crucible sealed. Analysis involved repeated heating and cooling scans from -40°C to 40°C at rates from 0.5°C/min to 10°C/min. Best results were obtained for 3°C/min scan rates.

Unilamellar Vesicle Preparation

Vesicles were made fresh daily. A liposome suspension was prepared by vortexing a lipid film (100  $\mu$ g of lipid and fluorescent probe to yield a >300:1, probe:phospholipid molar ratio) with degassed PBS, pH 7.4, at 37°C to make a 50x10<sup>-4</sup>M lipid solution. The sample was then sonicated for at least 10 minutes or until the mixture became clear to produce small unilamellar vesicles (20 nm diameter). Large unilamellar vesicles (100 nm diameter) were prepared in two ways: (1) by extruding a hydrated lipid mixture through a 100 nm filter more than 20 times, and (2) by a dialysis detergent removal technique in which phospholipid (and cholesterol, if required) was dissolved in cyclohexane, frozen, and cyclohexane sublimed away under vacuum. The resulting white, crystalline lipid was dissolved in a buffered octyl glucoside solution, and the octyl glucoside was then removed via dialysis. Stock solutions of phospholipids were routinely checked for purity by thin layer chromatography. Vesicle samples were also checked for lipid peroxidation after fluorescence analysis by observing the second derivative of the UV absorbance between 190 and 300nm, oxidized species appear at >230nm.

Microscopy

Model membrane systems were observed under a Zeiss Axiovert 45M microscope equipped with differential interference contrast, fluorescence optics, and temperature-controlled stage. A hydrated lipid film, compressed between a glass slide and coverslip, at a temperature greater than the main transition of the lipids, was used. Slide temperature was varied between 4°C and 60°C with a Peltier temperature controller. Micrographs of the samples were recorded on both polaroid and 35mm high contrast black and white film.

Fluorescence Analysis of Acyl Chain Packing

Measurements of DPH (1,6-diphenyl-1,3,5-hexatriene) fluorescence lifetime and anisotropy were made at 8 to 12 frequencies, log spaced from 2 to 100 MHz, with a frequency domain fluorometer. Excitation at 325 nm was provided by a He-Cd laser, emission was monitored after passage through a 400 nm cutoff filter. Total intensity decay was modeled as the sum of two discrete lifetimes:  $I_{\text{tot}}(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$ . Intensity-weighted average lifetime,  $\langle \tau \rangle$ , was calculated according to  $\langle \tau \rangle = (a_1 \tau_1^2 + a_2 \tau_2^2) / (a_1 \tau_1 + a_2 \tau_2)$ . Depolarization of DPH was characterized using a model based on a bimodal orientational distribution function,  $f(\theta)$ . Overall equilibrium ordering experienced by DPH was quantified by the free volume parameter,  $f_v$ , which characterizes the volume available for probe reorientational motion in the bilayer relative to that available in an unhindered, isotropic environment. The parameter  $f_v$  is defined by  $f_v = 1/(2f(\theta)_{\text{max}}) \int f(\theta) \sin(\theta) d\theta$ .

Major Findings:Effects of Polyunsaturation on DPH Orientational Distribution

Experiments were carried out to characterize the effects of unsaturation on acyl chain packing in protein-containing bilayers by measuring the dynamic anisotropy of DPH in a series of rhodopsin-containing vesicles consisting of di-14:0 PC, 16:0, 22:6 PC, and di-20:4 PC. Polyunsaturation greatly increases the fraction of DPH molecules oriented parallel to the bilayer. It was previously shown that  $f_v$  is correlated with the ability of rhodopsin to form MII. Polyunsaturation promotes a less cohesive acyl chain packing, corresponding to higher values of  $f_v$ . The value of  $f_v$  at 37°C in DMPC is attained in PDPC at 30°C and in DAPC at about 20°C. Thus, while saturated acyl chains have more intramolecular orientational degrees of freedom than polyunsaturated acyl chains, at physiological temperatures, polyunsaturated acyl chains yield a bilayer with lateral acyl chain packing properties more conducive to conformational changes of integral membrane proteins than do saturated acyl chains.

Phase Behavior of Polyunsaturated Lipids

DSC measurements of the gel to liquid-crystalline transition show that the gel-state of mixed-chain polyunsaturated PCs have minimal transition enthalpies for the 2 double bond species (<2 kcal/mole) but increasingly higher  $\Delta H$  for the higher polyunsaturates, with the highest  $\Delta H$  observed for the 22:6n3 species (6.5 kcal/mole). A preparative HPLC technique was developed, which yields large quantities of a synthetic preparation of 22:5n6 methyl esters separated from closely associated structures such as the trans isomer. A series of purified phospholipids, having a saturated fatty acid at the sn-1 position and 22:5n6 at the sn-2 position, was synthesized. This lipid species is of particular importance because there is a reciprocal replacement of 22:5n6 for 22:6n3 in n3 deficiency and this replacement coincides with deficits in neural function that are at least partially reversible with replenishment of 22:6n3. DSC was able to distinguish between 22:6n3 and 22:5n6 species based on their  $\Delta H$  values,  $\Delta H$  for the 22:5n6 species was 5.6 kcal/mole, significantly lower than that of the 22:6n3 species. These results are the first reported physical difference between these very similar lipids, and may partially explain the deficits resulting from the replacement of 22:5n6 for 22:6n3 in n3 deficiency.



The special packing properties of mixed-chain phospholipids containing a polyunsaturated acyl chain at the sn-2 position is thought to be due to the stronger saturated sn-1 chain interactions relative to the sn-2 chain interactions. In order to gain more insight into this question, a combination of DSC and microscopy was used to compare the phase behavior of 18:0, 22:6n3 PC to that of di-18:0 PC, di-22:6n3 PC, and a mixture of the two. The melting points for di-18:0 PC and di-22:6n3 PC are 55°C and -90°C, respectively. A mixture of these lipids show broad transitions at 40°C and at -90°C. These results suggest that the di-18:0 PC mixes with the di-22:6n3 PC, but a significant amount of the di-22:6n3 PC melts independently of the di-18:0 PC. This behavior is quite different from the mixed-chain 18:0, 22:6n3 PC where a single melt occurs at -5°C. At room temperature, micrographs of the di-18:0 PC contain gel-state structures including crystalline arrays, while the di-22:6n3 PC and 18:0, 22:6n3 PC have only lamellar structures. Micrographs of a mixture of di-18:0 PC and di-22:6 PC show crystalline arrays that are much larger and more organized than those in pure di-18:0 PC. Furthermore, an unrecognized lipid structure (possibly di-22:6n3 PC) accompanies these large crystalline arrays in significant quantity at regularly repeated crystalline defect points. These secondary structures have a much higher optical density than the crystalline areas and resemble tightly coiled "tubes". Unique polyunsaturate-induced structures observed for the di-18:0 PC/di-22:6n3 PC mixture suggest that unique organizational properties may exist for the mixed-chain species, since the saturate and polyunsaturate acyl chains cannot separate in mixed chain phospholipid as they do in the homogeneous chain systems. Such structures may be of size not observable with light microscopy. Fluorescence investigations, using phase selective probes, are planned to verify the composition and physical properties of these 22:6n3-dependent structures in both homogeneous and mixed-chain systems.

#### Effect of Ethanol on Phospholipid Acyl Chain Packing Properties

Fluorescence measurements of the dynamic anisotropy and lifetime behavior of DPH in 16:0, 18:1 PC and 16:0, 22:6 PC bilayer vesicles was used to determine the effect of ethanol on phospholipids acyl chain packing properties. Differential effects of ethanol in 16:0, 22:6 PC and 16:0, 18:1 PC bilayers were found with regard to both DPH average fluorescence lifetime,  $\langle \tau \rangle$ , and DPH fractional volume,  $f_v$ . Ethanol increased the values of both  $\langle \tau \rangle$  and  $f_v$  for both lipids, but induced a larger percentage increase in the 16:0, 22:6 PC parameters. These findings indicate that the effects of ethanol may be potentiated by the presence of polyunsaturated acyl chains. The high concentration of polyunsaturated acyl chains in the phospholipids of neurons and excitable tissue may make them particularly susceptible to the effects of ethanol.

#### Significance to Biomedical Research and the Program of the Institute:

Chronic alcohol exposure leads to the phenomenon of tolerance, in which membranes derived from animals exposed to ethanol no longer show the membrane disordering effect on acute alcohol exposure that is observed in membranes from control animals. This same effect is found in lipid extracts of these membranes, making it likely that alterations in lipid composition are responsible for this phenomenon. Our initial studies on the phospholipid acyl chain dependence of the effects of alcohol demonstrate that polyunsaturated acyl chains potentiate the effect of alcohol. This finding, coupled with the alcohol-induced depletion of 22:6n3 lipids shown by this laboratory, may provide an explanation for the development of the tolerance effect. Our studies of mixed-chain and homogeneous-chain polyunsaturated phospholipids reveal that mixed-chain molecules, which are the predominate motif in biological membranes, may provide membranes with a broader range of lateral organization than do phospholipids containing two identical fatty acids.



Proposed Course:

Our studies demonstrate that the presence of polyunsaturated acyl chains on phospholipid molecules induce special packing properties not present in membranes composed of symmetrically substituted phospholipids. In addition, the effects of ethanol are found to be more pronounced on phospholipids containing 22:6n3 than in similar phospholipids containing 18:1, suggesting that the effects of ethanol are potentiated by the presence of polyunsaturated acyl chains. These experiments will be expanded to include a graded variation in degree of unsaturation so as to better define the relationship between acyl chain content and the effects of ethanol on acyl chain packing. Fluorescence probes, which show a propensity for particular phases, will be used to explore the question of the formation of domains in membranes and bilayer vesicles as a consequence of the presence of polyunsaturated acyl chains. The effect of ethanol on the presence of both lateral and transmembrane domains will be determined in these experiments. The availability of 22:5n6-containing phospholipids will permit for the first time detailed comparison of the physical properties of phospholipids containing this acyl chain with those containing 22:6n3. Raman spectroscopy studies, which detected the increasing tendency of phospholipids to form microdomains as the level of unsaturation increased, will be extended to better characterize this phenomenon. Future studies will explore the effect varying headgroup (e.g., serine and ethanolamine) has upon the tendency of polyunsaturated phospholipids to form highly ordered structures in the gel-state and at physiological temperatures. Exploring these questions with a variety of membrane domain specific techniques will provide insight into the structural and functional role of polyunsaturated phospholipids in biological systems and aid in explaining the consequences of alcohol-induced, polyunsaturated fatty acid depletion.

Publications:

Niebylski CD, Salem N Jr. Time resolved fluorescence anisotropy and differential scanning calorimetry of a series of mixed-acid phosphatidylcholine bilayers: Effect of sn-2 acyl chain length and degree of unsaturation, *Biophys J* 1994;66(no.2, part 2):A56.

Niebylski CD, Salem N Jr. A calorimetric investigation of a series of mixed-chain polyunsaturated phosphatidylcholines: Effect of sn-2 chain length and degree of unsaturation, *Biophys J*, in press.

Salem N Jr, Niebylski CD. The nervous system has an absolute molecular species requirement for proper function, *Molecular Membrane Biol*, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 AA 00080-01 LMBB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Influence of Protein-Lipid Interactions on Signal Transduction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Litman Section Chief LMBB, NIAAA

Others: D. Mitchell Senior Staff Fellow LMBB, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Membrane Biochemistry and Biophysics

SECTION

Section of Fluorescence Studies

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

1.25

PROFESSIONAL:

0.75

OTHER:

0.50

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The integration of a large number of intracellular signals is mediated by G protein activating receptors in the plasma membrane of target cells. Goals of this project are to assess the role of polyunsaturated phospholipids in modulating G protein signal transduction and to elucidate the mechanism of action of ethanol and general anesthetics in these systems. The visual transduction pathway is being used as a model system. The effect of ethanol, anesthetics, and lipid composition on: the kinetics and extent of formation of metarhodopsin II (MII); MII Td complex formation; the rate of Td activation; cGMP PDE activation; and the GTPase activity of Td are being studied. Ethanol promotes the formation of MII, but is 40% more potent in a PDPC bilayer than in a POPC bilayer. Ethanol induces similar effects in both PDPC and ROS disk membranes, consistent with both these membranes having an sn-2 position containing almost exclusively 22:6n3 acyl chains. Alteration of the function of an integral membrane receptor, rhodopsin, via a phospholipid-mediated mechanism is most consistent with our results. The enhanced meta II formation produced by acute ethanol exposure would result in hyperactivity of this pathway, whereas the loss of unsaturated acyl chains in membrane phospholipids observed in chronic exposure to ethanol would be expected to diminish the effect of ethanol on the signalling pathway and may in this way contribute to the tolerance to ethanol developed in chronic ethanol exposure. Neutral salts, which reduce water activity, increase the potency of ethanol. The effect of water activity has generally been neglected, but clearly must be taken into account when investigating cellular processes in vitro. The kinetics of MII and MII Td formation were both faster POPC than in DMPC in the liquid crystalline phase. Increases in bilayer cholesterol markedly slows MII Td formation.

Project Description:Investigators:

B. Litman	Section Chief	LMBB, NIAAA
K. Hines	Biologist	LMBB, NIAAA
D. Mitchell	Senior Staff Fellow	LMBB, NIAAA

Objectives:

Major objectives of this project are to: (1) elucidate structure-function relationships in membranes containing polyunsaturated phospholipids and to determine what functionally critical properties these phospholipids impart to cellular membranes; and (2) obtain information on the functional requirements for lipids and the effect of ethanol and other perturbants, such as general anesthetics, on signal transduction.

Methods Employed:Preparation of ROS Disk Membranes and rhodopsin-containing vesicles

Bovine rod outer segments (ROS) and disks were purified following published procedures and stored at  $-70^{\circ}\text{C}$ . Rhodopsin was purified on a Concanavalin A affinity column. Large unilamellar vesicles containing rhodopsin were prepared by a dilution reconstitution method. All samples were prepared under red light illumination and handled and stored in an argon atmosphere.

Preparation of the ROS G protein, Transducin (Td)

Transducin (Td) was extracted from ROS by hypotonic washing. Specific GTP binding activity of Td in the final hypotonic extract was determined with a filter binding assay utilizing tritiated GMPPNP, a nonhydrolyzable GTP analogue.

Absorbance Spectra of MI  $\leftrightarrow$  MII Equilibrium

Corrected difference spectra of MI  $\leftrightarrow$  MII equilibrium mixtures were derived from a series of absorbance spectra acquired with a Hewlett-Packard 8452A diode array spectrophotometer. Individual MI and MII spectra were deconvolved from the equilibrium spectrum, using nonlinear least squares, and were used to calculate  $K_{eq}$ .

Kinetics of MII and MII•T Formation

Kinetics of MII and MII•T complex formation were derived from time-resolved absorbance changes at 380 nm, acquired with a laboratory-constructed flash photolysis instrument. Rate of decay of lumirhodopsin and rates of formation of MI and MII were calculated from the kinetics at 380 nm in terms of a specific microscopic rate model previously described.

Fluorescence Analysis of Acyl Chain Packing

Measurements of DPH (1,6-diphenyl-1,3,5-hexatriene) fluorescence lifetime and anisotropy were made with a frequency domain fluorometer, equipped with a He-Cd laser. Total intensity decay was modeled as the sum of two discrete lifetimes:  $I_{\text{tot}}(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$ . Intensity-weighted average lifetime,  $\langle \tau \rangle$ , was calculated according to  $\langle \tau \rangle = (a_1\tau_1^2 + a_2\tau_2^2) / (a_1\tau_1 + a_2\tau_2)$ . Depolarization of DPH was characterized using a model based on a bimodal orientational distribution function,  $f(\theta)$ . The motional properties of DPH were quantified by the free volume parameter,  $f_v$ , which characterizes the volume available for probe reorientational motion in the bilayer relative to that available in an unhindered, isotropic environment.  $F_v$  is defined by:  $f_v = 1/(2f(\theta)_{\text{max}}) \int f(\theta) \sin(\theta) d\theta$ .

Major Findings:The Effects of Ethanol on Receptor Activation:  $K_{eq}$  of MI  $\leftrightarrow$  MII

The effects of ethanol on  $K_{eq}$  in ROS disks, PDPC, and POPC were determined and expressed in terms of the additional change in free energy for MI  $\leftrightarrow$  MII,  $\Delta(\Delta G)$ , due to the presence of ethanol. The quantity  $\Delta(\Delta G)$  is defined by:  $\Delta(\Delta G) = \Delta G_{+EtOH} - \Delta G_{-EtOH}$ , where  $\Delta G = -RT \ln(K_{eq})$ , and  $\Delta G_{+EtOH}$  and  $\Delta G_{-EtOH}$  are the change in free energy in the presence and absence of ethanol, respectively. Ethanol decreases  $\Delta(\Delta G)$ , i.e., increases the equilibrium MII concentration, in all three bilayer systems. The slopes of the ethanol dose-response lines are  $586 \pm 34$ ,  $620 \pm 37$ ,  $442 \pm 27$  cal/mole/[ethanol] for rhodopsin in ROS disks, PDPC vesicles, and POPC vesicles, respectively. These results show that ethanol is about 40% more potent in a PDPC bilayer than in a POPC bilayer, whereas ethanol's effect on rhodopsin in PDPC and ROS disk membranes is identical, within experimental error, consistent with both their phospholipid sn-2 position containing almost exclusively docosahexaenoyl acyl chains. Ethanol had distinct effects on PDPC and POPC for both DPH average fluorescence lifetime,  $\langle \tau \rangle$ , and DPH free volume,  $f_v$ , indicating lipid specific changes in acyl chain packing properties. These results support a model in which ethanol alters the functioning of the integral membrane receptor rhodopsin via a phospholipid bilayer-mediated mechanism. In addition, the enhanced meta II formation produced by acute ethanol exposure would result in hyperactivity of this pathway. On this basis, the loss of unsaturated acyl chains in membrane phospholipids, which is observed in chronic exposure to ethanol, would be expected to diminish the effect of ethanol on the signalling pathway; this may contribute to the tolerance to ethanol developed in chronic ethanol exposure.

Potentiation of the Effect of Ethanol by Neutral Solutes

In a variety of studies, relatively high levels of ethanol were required to obtain measurable effects. In an effort to more closely simulate cytosolic conditions, where the activity of water is considerably lower than that in a dilute buffer solution, the dose-response behavior of  $K_{eq}$  with respect to ethanol was measured in the presence of a series of neutral solutes. The neutral solutes sucrose, stachyose, and PEG 400 (polyethylene glycol) lower the activity of water and enhance ethanol potency, showing increased levels of metarhodopsin II formation with increasing concentration of neutral salts. Once again the level of enhancement is greater in membranes containing polyunsaturated phospholipids. These results demonstrate that reduced water activity increases ethanol potency. This effect has generally been neglected in *in vivo* studies of ethanol's action on biological membranes, but clearly must be taken into account when attempting to investigate cellular processes *in vitro*.

Effects of Halothane and Octanol on  $K_{eq}$  of MI  $\leftrightarrow$  MII

In experiments to determine if general anesthetic action involves changes in G protein signalling pathways, the effects of octanol and halothane on  $K_{eq}$  of the MI  $\leftrightarrow$  MII equilibrium were examined in ROS disks at pH 7.0, 20°C. The results show that above a certain threshold concentration, both of these agents sharply reduce equilibrium MII concentration. The effect of octanol contrasts with that of ethanol (described above in first section of major findings) in that octanol, above 0.6-0.7 mM, inhibits MII formation, while ethanol shows a continuous enhancement of MII formation, with increasing ethanol concentration. These findings demonstrate that the effects of alcohols on integral membrane receptor conformation changes depend on alcohol chain length. A chain length dependent effect of alcohols is consistent with these molecules binding at the interfacial region of the bilayer and the perturbation of bilayer packing properties being dependent on the depth of penetration of the alcohol alkyl chain into the bilayer.

Effects of Bilayer Composition on Rate of MII and MII•Td Formation

The first step in signal transduction after the active receptor conformation forms is the formation of a receptor•G protein complex. Photoactivation of rhodopsin in the presence of G protein and absence of GTP leads to the formation



of a stable MII•Td complex. In a flash photolysis experiment, the rate of MII formation and MII•Td complex formation can both be determined. The presence of 30 mol% cholesterol in egg PC vesicles increased the time constant of MII formation by 14%, from  $10.6 \pm 0.4$  to  $12.1 \pm 0.3$  ms (at 20°C), and increased the time constant for MII•Td complex formation by 40%, from 100 to 140 ms. These results show that bilayer cholesterol somewhat impedes integral membrane conformational change, and greatly slows the 2-dimensional reaction of MII and Td to form the MII•Td complex. The effects of acyl chain unsaturation on the kinetics of MII and MII•Td formation were examined by comparing the rates of these processes for rhodopsin in DMPC and POPC vesicles. At 30°C both of these bilayers are in the liquid crystalline phase, and both processes were faster in POPC. In DMPC the time constants of MII and MII•Td formation were  $8.3 \pm 1.0$  and  $74 \pm 6$  ms, respectively, while in POPC they were 20% ( $6.6 \pm 0.6$  ms) and 30% ( $52 \pm 4$  ms) faster, respectively. These results show that increases in bilayer cholesterol and decreases in acyl chain unsaturation markedly slow a 2-dimensional bimolecular reaction process that is an essential step in signal transduction.

#### Significance to Biomedical Research and the Program of the Institute:

Current proposed mechanisms of alcohol and general anesthetic action emphasize the modulation of integral membrane protein function by either the indirect effect of anesthetic alteration of membrane bilayer properties or by the direct binding of anesthetics to proteins. Of critical importance in nerve conduction is the integration of signals resulting from the release of neurotransmitters, which act as agonists for receptors in the plasma membrane of target cells. Thus, proteins involved in signal transduction pathways are likely the targets of anesthetic action. G protein-mediated signal transduction plays a major role in neurotransmitter-associated signalling as well as the regulation of many other membrane-associated functions. These pathways involve the coupling of extracellular signals sensed via plasma membrane receptors with their effector protein by a guanine nucleotide binding protein or G protein. These receptors belong to a large family of integral membrane proteins, which share the seven transmembrane helical structural motif, as well as a relatively high level of sequence homology. Thus, observations made on any one of these systems will be relevant to the function of this ubiquitous receptor family. The visual transduction pathway is unique in that individual steps in the signalling pathway can be studied quantitatively, thus allowing questions relative to the mechanism of action of ethanol and general anesthetics to be answered in a definitive way. In addition, the ability to reconstitute the pathway in synthetic phospholipid vesicles of defined lipid composition allows one to define the role of various membrane components in potentiating the effects of ethanol, general anesthetics, and lipid soluble drugs. The results of these studies can lead to a better understanding of the relationship between essential fatty acid nutrition and the efficacy of various membrane perturbing agents, the design of more effective anesthetics, and an explanation of ethanol tolerance in chronic alcohol exposure.

#### Proposed Course:

Current experiments on the effect of ethanol on signal transduction and the role of membrane lipid composition in mediating these effects will be extended. The composition of reconstituted systems used in these experiments will be varied further to better define the effects of phospholipid acyl chain and headgroup composition and cholesterol content. Signal transduction in ROS disks derived from animals maintained on several controlled dietary regimes, such as low essential fatty acids and controlled alcohol exposure, will be studied in order to correlate our studies in reconstituted systems with native membranes. The variation on lipid composition induced by the restricted dietary conditions will be determined, allowing a correlation between any observed change in function of the signalling pathway and membrane composition. In addition to continuing our studies on receptor activation, G protein diffusional search, G protein-receptor complex formation, G protein activation, and effector protein activation will be

studied to obtain a full picture of the steps in signalling subject to alteration by ethanol and general anesthetics and the variation of lipid composition. Where possible, experiments will be carried out on lipid and detergent free preparations. In this way, both the lipid-mediated and direct protein binding mechanisms of ethanol and general anesthetic action will be tested. The importance of the activity of water in potentiating the effects of ethanol will be further examined to determine the effect of neutral solvents on the efficacy of ethanol. The presence of polyunsaturated phospholipid acyl chains appears to promote both the formation of active receptor and the tendency to form lateral domains. Future experiments will examine the role of membrane domains in modulating various steps in the signalling pathway.

#### Publications:

Mitchell DC, Litman BJ. Effect of ethanol on metarhodopsin II formation is potentiated by phospholipid polyunsaturation, *Biochemistry*, in press as an accelerated publication.

Mitchell DC, Litman BJ. Effect of ethanol on receptor conformation change: Phospholipid acyl chain unsaturation augments ability of ethanol to enhance both meta II formation and acyl chain packing free volume, *Biophys J* 1994;66(no. 2, part 2):A48.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00284-05 LMBB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alterations in Lipid Metabolism in the Nervous System by Ethanol

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	H-Y. Kim	Section Chief	LMBB, NIAAA
Others:	M. Garcia	Visiting Fellow	LMBB, NIAAA
	Y-C. Ma	Visiting Fellow	LMBB, NIAAA
	F. Thies	Visiting Fellow	LMBB, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Membrane Biochemistry and Biophysics

SECTION

Section of Mass Spectrometry

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

5.0

PROFESSIONAL:

4.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects   ☐ (b) Human tissues   ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The principal objective of this study is to elucidate metabolic functions of polyunsaturated fatty acids and phospholipids in nervous tissues with particular reference to their modulation by ethanol. Our studies focused on incorporation, release, and remodeling of the major polyunsaturates in the brain, docosahexaenoic acid (22:6n3) and arachidonic acid (20:4n6). Active incorporation of both 20:4n6 and 22:6n3 into rat brain occurred during the first month of life. Enrichment of 22:6n3 in comparison to 20:4n6 occurred during this stage. Incorporation of 20:4n6 appeared to involve more extensive remodeling processes in comparison to 22:6n3 in developing rat brain. Total incorporation of 22:6n3 and 20:4n6 into adult brain was similar.

Endogenous synaptosomal PLA2 appeared to selectively hydrolyze 20:4n6 and maintain 22:6n3 under normal conditions. The basal PLA2 activity was unaltered by ethanol in synaptosomes; however, the PLA2 activity stimulated by ionophore was inhibited with 0.1% ethanol treatment in vitro, suggesting that acute ethanol exposure may affect the PLA2 activation process. Chronic ethanol exposure elevated the free fatty acid level in the brain significantly. Incorporation of 20:4n6 and 22:6n3 was slightly inhibited, partly explaining the elevated free fatty acid level observed after ethanol treatment. These results suggest that ethanol tends to decrease the polyunsaturated fatty acid turnover in neuronal membranes. In contrast to the synaptosomal PLA2 activity, both 20:4n6 and 22:6n3 were released from C-6 glioma cells. Serotonin and norepinephrine stimulated the release of both fatty acids, indicating that the release of polyunsaturated fatty acids involved receptor-mediated processes. The polyunsaturated fatty acid profile was easily modified in C-6 glioma cells, providing an excellent model to examine the effect of acyl modification on phospholipid remodeling processes.

Project Description:Investigators:

H-Y. Kim	Section Chief	LMBB, NIAAA
L. Edsall	Chemist	LMBB, NIAAA
M. Garcia	Visiting Fellow	LMBB, NIAAA
Y-C. Ma	Visiting Fellow	LMBB, NIAAA
F. Thies	Visiting Fellow	LMBB, NIAAA

Objectives:

The major objectives of this project are to: (1) delineate the underlying metabolic mechanisms for concentrating and maintaining long chain polyunsaturated fatty acids, especially 20:4n6 and 22:6n3, in the nervous system, and to describe the effect of ethanol on these processes; and (2) describe the phospholipid remodeling processes affected by ethanol and the polyunsaturate profile.

Methods Employed:Release of Polyunsaturated Fatty Acids

Cytosol and/or synaptosomes prepared using the sucrose gradient method were incubated at 37.5°C for 0-60 minutes in the presence of 50  $\mu$ M CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, and 50  $\mu$ M ATP. In some cases, various neurotransmitters, calcium ionophore, and calcium blockers were coinubated in order to characterize the mechanism of the polyunsaturated fatty acid release. The reaction was terminated by acidification to pH 3.0 with formic acid, and the lipids were extracted according to the Bligh and Dyer method in the presence of 23:0 as an internal standard. An aliquot was transmethylated for reference and the rest was applied to an aminopropyl solid phase extraction column. Isolated free fatty acids as well as other lipid classes were subjected to GC analysis after transmethylation.

Release of Polyunsaturated Fatty Acids from C-6 Glioma Cells

C-6 glioma cells were plated in 9 cm<sup>2</sup> wells at an initial density of 5 x 10<sup>4</sup> cells using DMEM (Dulbecco's Modified Eagle Medium) supplemented with 5% (v/v) fetal bovine serum, penicillin (100 Units/mL), and streptomycin (100  $\mu$ g/mL). Tritiated 20:4n6 and <sup>14</sup>C-22:6n3 (0.5  $\mu$ Ci each, adjusted to 50  $\mu$ Ci/ $\mu$ mole) were added to the medium and incubated for 48 hours. The cells were washed twice with DMEM containing 0.2% fatty acid free bovine serum albumin and 20 mM Hepes. The cells were incubated with 1 mL of the same medium in the presence or absence of agonists at 37°C and the appearance of the radioactivity in the supernatant was followed to 30 min. The identity of the radioactive compounds in the supernatant was determined by TLC (hexane:diethyl ether:acetic acid 90:20:2) after lipid extraction according to Bligh and Dyer.

Assay for Serine Exchange Reaction

C-6 glioma cells were cultured in 30 mm dishes with DMEM supplemented with 5% (v/v) fetal bovine serum, penicillin (100 Units/mL), and streptomycin (100  $\mu$ g/mL). At confluence, the medium was replaced with a serine-free medium and cells were incubated with <sup>3</sup>H-serine (1.5  $\mu$ Ci, 20 Ci/mmmole). The cells were harvested at various incubation times, lipids were extracted by the Bligh and Dyer method, and the lipid phase was carefully washed with methanol and PBS buffer in a 1:9 ratio (v/v). The lipid extracts were analyzed by TLC with chloroform:methanol:acetic acid:water (100:75:7:4) as the developing solvent. Separated TLC bands were scraped and radioactivity was counted using a liquid scintillation counter.

Phospholipase D Assay

Phospholipase D (PLD) activity was assessed by measuring the synthesis of <sup>3</sup>H-phosphatidylbutanol from <sup>3</sup>H-butanol. Confluent cells were preincubated with <sup>3</sup>H-butanol (5  $\mu$ Ci, 15 Ci/mmmole) for 15 minutes prior to the addition of agonists. At various time points, the incubation was terminated by adding ice-cold methanol



and carrier phosphatidylbutanol. Lipids were extracted and separated by TLC using a solvent system comprising the organic phase of 2,2,4-trimethylpentane:ethyl acetate:acetic acid:water (50:110:20:10). Separated bands were scraped and radioactivity was counted by liquid scintillation.

#### Analysis of Fatty Acid Incorporation

Sprague-Dawley rats of various ages (0-21 days old) were sacrificed and the fatty acid content in the brain, liver, plasma, and stomach was determined. Alternatively, these animals were fed radiolabelled 20:4n6 and/or 22:6n3. Incorporation of radioactivity into brain and liver and the clearance of radioactivity from the stomach contents were followed at 2, 6, 12, and 24 hours after feeding. Lipids were extracted and analyzed using TLC/liquid scintillation or by HPLC/radioactivity detector. *In vitro* incorporation studies were performed using rat brain homogenate and brain subcellular fractions. Radiolabelled 20:4n6 and 22:6n3 were incubated for 20 minutes at 37°C with appropriate tissue samples in 75 mM Tris-HCl buffer (pH 8.0) containing 4 mM MgCl<sub>2</sub>, 55 μM CoA, and 100 μM ATP in the presence of 0-1% ethanol.

#### Phospholipid Molecular Species Analysis

Using an HP Hypersil-ODS column (20 cm x 2.1 mm ID, 5μ) with a mobile phase of water:0.5% NH<sub>4</sub>OH in methanol:hexane changing from 10:90:0 to 0:90:10 in 17 minutes after holding at the initial composition for three minutes, separation of PS, PI, PE, and PC was achieved in 20 minutes. The flow rate was 0.5 mL/min. The effluent was split 1:100 using a commercial splitter (LC Packing) to deliver 5 μL/min to the needle of an HP electrospray source mounted on a Hewlett-Packard 5989 mass spectrometer. The capillary, ground, and cylinder potentials were set at -4500, -3500, and -3000V, respectively. For thermospray LC/MS analysis, the HPLC effluent together with the additional flow of 0.4% CH<sub>3</sub>COOH in water at a flow rate of 0.6 mL/min was directly introduced into an Extrel ELQ-400 mass spectrometer via Vestec thermospray interface. The source and vaporizer tip temperatures were set at 280°C and 210°C, respectively.

#### Major Findings:

##### Release of Polyunsaturated Fatty Acids from Rat Brain Synaptosomes

Previously, we observed that 20:4n6 was preferentially released from rat brain synaptosomes upon incubation at 37°C. In contrast to the hydrolysis of synaptosomes by cobra venom Phospholipase A<sub>2</sub>, where the hydrolysis of 22:6 is most prominent, release of 20:4n6 was consistently higher than that of 22:6n3 and 18:1n9. Similar activity was observed from the brain cytosolic fraction. According to the thermospray and electrospray LC/MS analyses, the released 20:4n6 fatty acid appeared to primarily originate from 16:0, 20:4-phosphatidylinositol (PI) and 18:0, 20:4-PE. The apparent preferential hydrolysis was not due to the selective reincorporation of the released fatty acids into phospholipids, since reincorporation of released free fatty acids were negligible under the employed incubation conditions.

Extracellular calcium concentration from 0.1 μM-10 mM did not affect the hydrolysis activity significantly. Even in the presence of ionophore, only a slight increase of fatty acid release was observed. Chelation of calcium by EGTA did not alter the hydrolysis profile, suggesting the hydrolysis of 20:4n6 from synaptosomes may be a calcium independent process. Phorbol myristate stimulated the release of 20:4n6 significantly from both synaptosomes and cytosol, suggesting that protein kinase C activation is involved in the release of polyunsaturated fatty acids.

In order to further understand the mechanism of 20:4n6 release from synaptosomes, the polyunsaturated fatty acid profile in diacylglycerol (DG) was examined in comparison to that in the free fatty acid (FFA) fraction. After 30 minutes of incubation, 20:4n6 was increased in both FFA and DG fractions; increase in FFA was higher than in DG. Only a slight increase in the 22:6n3 level was observed in DG. The level of 20:4n6 containing DG obtained after 30 minutes of incubation

in the presence of ETYA was reduced to the zero minute control level while the production of 20:4n6 FFA was only slightly inhibited, indicating that the changes in 20:4n6 level in FFA and DG did not occur concomitantly. These results suggest that free fatty acid release observed from rat brain synaptosomes resulted primarily from PLA<sub>2</sub> activity rather than PLC and di- and monoacylglycerol lipase activity.

Chronic ethanol treatment for 20 days did not alter either the endogenous phospholipase A<sub>2</sub> activity or synaptosomal membrane sensitivity towards exogenous PLA<sub>2</sub> activity. Acute ethanol exposure at 0.1% ethanol did not affect basal PLA<sub>2</sub> activity; however, PLA<sub>2</sub> activity stimulated by ionophore was slightly but significantly inhibited. Both basal and ionophore-stimulated PLA<sub>2</sub> activity was inhibited in the presence of 1% ethanol.

#### Polyunsaturated Fatty Acid Metabolism in C-6 Glioma Cells

Subcellular fractionation of the brain homogenate does not produce synaptosomal preparations entirely from neuronal origin. Attachment of glial cells is the most common non-neuronal contamination observed from these preparations. In order to assess the contribution to free fatty acid release from glial cell contamination, we investigated C-6 glioma cells after labeling with 20:4n6 and 22:6n3. In contrast to the preferential 20:4 release in synaptosomes, both 20:4n6 and 22:6n3 were similarly hydrolyzed at the basal level as well as after stimulation in C-6 cells. Therefore, the specificity observed for 20:4n6 in synaptosomes may not be due to glial cell contamination. In this system, treatment with calcium ionophore stimulated the release of both 20:4n6 and 22:6n3. In addition, neurotransmitters such as serotonin and norepinephrine stimulated the release by three-four fold, indicating that receptor-mediated processes are involved in the free fatty acid release. After stimulation, 20:4 and 22:6 containing diacylglycerols were also increased in the medium, suggesting that phospholipase C and diacylglycerol lipase as well as phospholipase A<sub>2</sub> reactions may be involved in receptor-mediated fatty acid mobilization.

Serine exchange as well as phospholipase D activity was also observed in C-6 glioma cells. The radioactivity from serine steadily increased in the PS fraction and was sustained up to four hours. Since phosphatidic acid, the product of PLD, is not metabolically stable, transphosphatidylation to stable phosphatidylbutanol was used to assess PLD activity. Treatment with phorbol myristate increased the formation of phosphatidylbutanol significantly in comparison to the control at all incubation time points examined up to 30 minutes, indicating that PLD can be stimulated in C-6 glioma cells.

Alteration of the lipid profile may affect the fatty acid release through changing receptor or enzyme activity. Lipid profile of C-6 glioma cells could be manipulated by adding 20:4n6 and/or 22:6n3 to the culture medium of rapid growing cells. Without supplementation, glioma cells contain a very low level (2-3% of total lipid) of 22:6n3. By 24 hours of incubation at 100  $\mu$ M, 20:4n6 or 22:6n3 was incorporated up to 20% of the total fatty acyl composition. According to the phospholipid molecular species analysis by electrospray LC/MS, it was apparent that 22:6n3 is taken up into PA, PI and PC first, probably through either *de novo* synthesis or deacylation/reacylation reaction, and then remodeled to PE and PE-plasmalogens in C-6 glioma cells. In contrast, the 22:6-containing PS accumulated during the first 24 hours did not appear to undergo significant remodeling. Using this model, the effect of ethanol and polyunsaturate profile on receptor-mediated fatty acid release, serine exchange, and phospholipase D reaction is now under investigation.

#### Effect of Ethanol on the Level of Brain Free Fatty Acids

Analysis of microwave-killed rat brain indicated that the basal free fatty acid distribution resembles that of total lipids. Chronic ethanol treatment for 77 days increased the basal free fatty acid level in rat brain. However, the fatty acyl distribution was not altered significantly, indicating that this phenomenon was probably not due to the stimulation of PLA<sub>2</sub> activity. The presence of 0.5-1%

ethanol slightly decreased *in vitro* incorporation of both 20:4n6 and 22:6n3 in rat brain homogenate and microsomes, suggesting that ethanol elevates the basal free fatty acid level possibly through inhibition of the acylation process. In contrast to the basal free fatty profile, the level of free fatty acid observed after ischemic decapitation was considerably higher than the basal level and accompanied by a prominent elevation of the 20:4n6 proportion. These observations may indicate that under stimulated conditions, phospholipase activity is a major factor in controlling the free fatty acid profile, while acyltransferase activity is a more important contributor in the resting state.

#### Incorporation of Polyunsaturated Fatty Acids into Brain

In order to understand the mechanism whereby 22:6n3 is enriched in the brain, we investigated the incorporation of this fatty acid in comparison to 20:4n6 with newborn rats, since brain growth is expected to be most active at this stage. Previously, we have found that the absolute level of both 22:6n3 and 20:4n6 linearly increased in the brain during the first month after birth, whereupon the incorporation leveled off. We also found that preferential incorporation of 22:6n3 with respect to 20:4n6 occurs prominently during 7-21 days after birth and the total incorporation profile becomes similar 40 days after birth. Total incorporation of radioactive 20:4n6 and 22:6n3 into adult rat brains did not show any significant differences when they were injected into the brain ventricle. Similar results were obtained from *in vitro* incubation with adult brain homogenate.

During the first month of life, *in vivo* incorporation profiles for 20:4n6 and 22:6n3 after feeding were similar. Although radioactivity from 22:6n3 was slightly higher than that of 20:4n6 in brain during the first hour after feeding, the level of 20:4n6 became similar to that of 22:6n3 by 24 hours. Analysis of radioactivity in the individual phospholipid classes indicated that the incorporation process for these two fatty acids was quite different; 20:4n6 was incorporated into brain phospholipids via more diversified metabolic pathways involving efficient turnover from neutral lipids to PI/PS and PC and then subsequently to PE, whereas 22:6n3 was directly incorporated into PE without efficient remodeling. Since the total radioactivity incorporated into brain was similar for both fatty acids, it is not clear how enrichment of 22:6n3 in comparison to 20:4n6 occurred during the first month of the life. It is possible, however, that a time course limited to 24 hours may not be sufficient in fully assessing the fate of ingested 20:4n6 and 22:6n3. It is also speculated that inefficient turnover of 22:6n3-containing lipids may help to maintain the high content of this fatty acid in brain.

#### Polyunsaturated Phospholipid Turnover

After 22:6n3 is preferentially incorporated in the brain during development, it appears to be relatively well maintained throughout life. Our results from PL<sub>A</sub> studies showed that 22:6n3 is neither the preferred substrate nor preferentially incorporated in comparison to 20:4n6 in adult rat brain as assessed by *in vitro* incubation and *in vivo* intraventricular injection or feeding of 20:4 and 22:6 fatty acids. These data suggested that turnover of 22:6 fatty acid may be a slow process. Ethanol may alter the polyunsaturate content through altering the turnover process of specific phospholipid molecular species. In order to test this hypothesis, brains of pups were labeled by feeding pregnant mothers with d5-18:3n3 and d5-18:2n6 during pregnancy and the lactation period. All the molecular species containing 20:4n6 and 22:6n3 were labeled to an equal extent, indicating that representative labeling was obtained. After labeling, turnover of 20:4 and 22:6 species was monitored by feeding unlabeled fatty acids in order to determine whether specificity exists in the deacylation and reacylation process. Ethanol effect on this process is under investigation.

#### Phospholipid Molecular Species Analysis

The efforts to develop a fast and convenient method to analyze phospholipid molecular species continued in this period. Based on the reversed phase HPLC technique previously developed in our laboratory for molecular species analysis



for thermospray LC/MS, an improved technique for phospholipid molecular species analysis was developed. Unlike the thermospray technique, phospholipid molecular species were separated and detected mainly as protonated or natriated molecular species. The response was linear over two orders of magnitude, allowing quantification of each molecular species. Marked improvement in sensitivity was also observed. The present quantification limit is approximately 0.2-0.5 pmole before split (2-5 fmole after 1/100 split). The relative responses were more dependent on the headgroup identity rather than fatty acyl composition within a phospholipid class. This technique is now being applied to the quantification of molecular species in lipid remodeling studies.

#### Significance to Biomedical Research and the Program of the Institute:

It has been proposed that an important mechanism underlying many of the effects of ethanol is its capacity to alter polyunsaturated metabolism. Alterations in processes involving incorporation, release, and remodeling of long chain polyunsaturates, which are especially enriched in brain, may be related to neuronal dysfunction caused by ethanol. Our studies indicated that active incorporation of both 20:4n6 and 22:6n3 into rat brain occurs during the first month of life, indicating the importance of the nutritional supply at this early developmental stage for the accretion of polyunsaturates in nervous system. One of the effects of ethanol on polyunsaturated fatty acid metabolism appeared to be the decrease in polyunsaturated fatty acid turnover in neuronal membranes as indicated by the inhibition of polyunsaturated fatty acid incorporation and synaptosomal PLA<sub>2</sub> activity during activation. The basal free fatty acid level in brain, elevated by chronic ethanol exposure, may affect brain functions. The different hydrolysis profiles observed in synaptosomes and C-6 glioma cells may indicate the distinctive role of polyunsaturated fatty acid release in these systems. Receptor-mediated polyunsaturated fatty acid release as observed in glioma cells may be modified by ethanol. Due to the easily modifiable polyunsaturate profile, as well as active lipid metabolism in C-6 glioma cells, this cell line may provide a valuable model to investigate the effect of ethanol and polyunsaturates on phospholipid remodeling processes in neuronal systems.

#### Proposed Course:

We will continue our characterization of metabolic pathways of polyunsaturates in the nervous system. We will continue to study incorporation, release, and remodeling of polyunsaturates in the brain and the effect of ethanol on these processes. Investigation on preferential metabolism of phospholipid molecular species will continue, using radioactive precursors or deuterated lipid standards and mass spectrometry. Newly developed LC/MS methodology will be applied to elucidate mechanisms of fatty acid release and phospholipid remodeling. Lipoygenation in pineal, its modulation by ethanol as well as the biological effect of the resulting metabolites, will be investigated. Oxygenation of phospholipids will be investigated in relation to its formation in target organs and their elaboration by ethanol.

#### Publications:

Garcia MC, Shigekawa M, Nakanishi S, Ito S. Multiple mechanisms of arachidonic acid release in Chinese hamster ovary cells transfected with cDNA of substance P receptor, *Biochem Pharmacol*, in press.

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Kim HY, Salem N Jr. Liquid chromatography-mass spectrometry of lipids, *Progress in Lipid Research* 1993;32:221-45.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00003-02 LMBB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

NMR Investigations of Cell Membrane Structure

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. Gawrisch	Visiting Scientist	LMBB, NIAAA
Others:	J. Barry	Senior Staff Fellow	LMBB, NIAAA
	L. Holte	NRC Fellow	LMBB, NIAAA
	F. Separovic	Visiting Fellow	LMBB, NIAAA
	T. Sinnwell	Chemist	LMBB, NIAAA

COOPERATING UNITS (if any)

LBC, NHLBI (J.A. Ferretti, B. Koenig); NIST (S. Krueger); Princeton U (S. Erramilli, S. Peter); Hebrew U (L. Bergelson); LG NCI (S. Janz); FDA (D. Lester)

LAB/BRANCH

Laboratory of Membrane Biochemistry and Biophysics

SECTION

Section of Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

4.5

PROFESSIONAL:

3.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objectives of this project are to: (1) investigate the interaction of alcohol with proteins and lipids in biological membranes; (2) study structure and dynamics of membranes composed of lipids with polyunsaturated fatty acids such as docosahexaenoic acid (DHA, 22:6n3); and (3) study lipid-protein interaction related to alcoholism and lipid polyunsaturation. Our goal is to identify general biophysical properties which are important for the behavior of membranes found in biological tissues. Alcohol-protein/lipid interaction: The mechanisms underlying the membrane-mediated effects of ethanol were examined via the interaction of ethanol with phospholipid bilayers at hydration levels of 10-12 water molecules per lipid. Bound ethanol has an order parameter comparable to the order in a lipid headgroup. Ethanol binding altered the orientation of the lipid headgroups and disordered the acyl chains of lipids, as evidenced by reductions in the deuterium NMR order parameters of the chains. It was concluded that ethanol interacts with phospholipid bilayers at the lipid-water interface (consisting of the headgroup, glycerol backbone, and uppermost chain methylene groups), rather than in the hydrocarbon interior. Lipid-lipid interaction: The temperature dependence of the chain order parameter profile for a series of phosphatidylcholines (PC) with perdeuterated stearic acid, (18:0), in position sn-1 and 18:1n9, 18:2n6, 18:3n3, 20:4n6, 20:5n3, or 22:6n3 in position sn-2 was investigated. The chain length of the sn-1 chain decreases with increasing unsaturation of the sn-2 chain and increasing temperature. Highly unsaturated PCs had shorter chains but the chain length was less sensitive to temperature changes. At 37 C, the area per lipid molecule in the bilayer increased from 65 Å<sup>2</sup> for (18:1n9) to 70 Å<sup>2</sup> for (22:6n3). Protein-lipid interaction: The conformation, dynamics, and binding to membranes of the peptide fragment 828-848 with the sequence R V I E V V Q G A C R A I R H I P R R I R from the carboxy-terminal region of the envelope glycoprotein gp41 of HIV-1 were investigated by NMR. The peptide forms a flexible helical structure upon binding to negatively charged membranes. The helix is oriented parallel to the bilayer surface.



Project Description:Investigators:

K. Gawrisch	Visiting Scientist	LMBB, NIAAA
J. Barry	Senior Staff Fellow	LMBB, NIAAA
L. Bergelson	Professor	Hebrew U
J. Ferretti	Section Chief	LBC, NHLBI
L. Holte	NRC Fellow	LMBB, NIAAA
S. Janz	Visiting Scientist	LG, NCI
B. Koenig	DAAD Fellow	LBC, NHLBI
S. Krueger		CNRF, NIST
D. Lester		DRT, CDER, FDA
S. Peter	Undergraduate Student	Princeton U
F. Separovic	Visiting Fellow	LMBB, NIAAA
T. Sinnwell	Chemist	LMBB, NIAAA

Objectives:

The major objectives of this project are to: (1) investigate the interaction of alcohol with proteins and lipids in biological membranes and the resulting alterations in the self-organization of membranes; (2) study structure and dynamics of membranes composed of lipids with polyunsaturated fatty acids such as docosahexaenoic acid (DHA, 22:6n3) so that their functional roles may be assessed; and (3) study lipid-protein interaction related to alcoholism and lipid polyunsaturation.

Methods Employed:

The structure of biological membranes is investigated by solid-state NMR techniques, primarily using deuterium labeling. Data are analyzed in terms of order parameters of chemical bonds of lipids and proteins. In collaboration with other laboratories, we perform multidimensional high resolution NMR, x-ray diffraction, neutron and x-ray reflectometry, calorimetry, fluorescence spectroscopy, and electrical surface potential measurements on the same systems. Structural information is combined with thermodynamical information such as changes in free energies, shifts in phase transition temperatures, changes in transition enthalpies, and association constants. Experimental results are summarized in models which highlight functional aspects of membranes related to alcoholism and fatty acid unsaturation.

Major Findings:Alcohol-Protein/Lipid Interaction

The mechanisms behind the membrane-mediated effects of ethanol were examined via the interaction of ethanol with phospholipid bilayers at hydration levels of 10-12 water molecules per lipid. At this water concentration the lipid headgroups are fully hydrated but no excess water is present. The measurements reflect the influence of ethanol on bilayer structure for a given mole ratio of ethanol per lipid, almost unperturbed by differences in partitioning between the water phase and the membrane. Deuterium and phosphorus NMR spectroscopy were used to monitor deuterated water and ethanol, and the headgroups and acyl chains of neutral phospholipids. Ethanol was found to interact strongly with both phosphatidylcholine and phosphatidylethanolamine bilayers, giving deuterium NMR quadrupolar splittings for the deuterated methylene group of ethanol between 6.3 and 9.4 kHz. The quadrupolar splittings for ethanol in gel phase lipids remained well resolved and were not significantly larger than those in the liquid crystalline lamellar phase, suggesting that little or no ethanol was bound in the hydrocarbon interior of the bilayer. Ethanol binding altered the orientation of the lipid headgroups, as shown with headgroup-deuterated PC bilayers. The entire length of the acyl chain was disordered by the ethanol interaction, as evidenced by significant reductions in the deuterium NMR order parameters of the chains.

The disordering corresponds to an increase in the area per lipid by an estimated 6% with one ethanol molecule per lipid, and a total of 18% with a second ethanol per lipid. This pronounced area increase is presumably caused by the disruption of lipid packing in the rigid region of the glycerol backbone rather than the acyl chains, since the order of the hydrocarbon chains is not affected to a significant degree by incorporation of alkanes and long chain alcohols into the hydrocarbon interior. From these data it was concluded that ethanol interacts with phospholipid bilayers primarily at the lipid-water interface (consisting of the headgroup, glycerol backbone, and uppermost chain methylene groups), rather than in the hydrocarbon interior. The lipid-ethanol interaction described here may serve as a model for the binding of ethanol in the hydrophobic-hydrophilic interface regions of macromolecules in general, including membrane proteins and carbohydrates.

#### Lipid-Lipid Interaction

The objective of these experiments is to correlate lipid polyunsaturation to changes in the order parameter profile of phospholipids and to the lipid bilayer thickness, area per molecule, and phase transition temperatures of the lipid matrix. Solid-state deuterium NMR spectroscopy was used to study the temperature dependence of the quadrupolar splittings for a series of phosphatidylcholines with perdeuterated stearic acid, (18:0), in position sn-1 and 18:1n9, 18:2n6, 18:3n3, 20:4n6, 20:5n3, or 22:6n3 in position sn-2. All lipids form crystalline lamellar phases at low temperature and liquid crystalline lamellar phases at higher temperature. Analysis of the spectral first moments showed temperatures for transition between the two phases upon warming of 4.8°C, -12.6°C, -10.8°C, -13.5°C, -12.6°C, and -2.0°C, respectively. A hysteresis between warming and cooling run was observed for 18:2n6 (5.2°C), 18:3n3 (2.2°C), and 22:6n3 (11.1°C). The order parameter profiles of chain methylene segments were calculated from dePaked NMR powder patterns. Comparison of the profiles at 37°C showed that increasing sn-2 chain unsaturation resulted in a 1.6 kHz decrease in quadrupolar splittings of the sn-1 chain in the upper half of the chain (or plateau region) and maximal decrease of 4.4 kHz near the middle of the chain at methylene carbon 13. This decrease in chain order corresponds to a change in length of the hydrocarbon chain. At 37°C, a decrease in the 18:0 chain length of  $0.4 \pm 0.2$  Å was observed when 18:2 versus 18:1 was in position sn-2. Fatty acids containing three or more sn-2 double bonds resulted in a decrease in sn-1 chain length of  $0.7 \pm 0.2$  Å. The chain length of all lipids decreased with increasing temperature over the investigated temperature range (0-47°C). Highly unsaturated phosphatidylcholines (three or more double bonds in sn-2) had shorter sn-1 chains but the chain length was somewhat less sensitive to temperature. Using the assumption that sn-1 and sn-2 chains have the same length in the liquid crystalline lamellar phase, the mean areas per lipid molecule were calculated. At a temperature of 37°C the area per lipid increased with increasing number of double bonds in the sn-2 chain from  $65 \text{ Å}^2$  (18:1n9) to  $70 \text{ Å}^2$  (22:6n3). Headgroup conformation and mobility of all lipids were identical as assessed by phosphorus NMR spectroscopy.

#### Protein-Lipid Interaction

The conformation, dynamics, and binding to membranes of the peptide fragment 828-848 with the sequence R V I E V V Q G A C R A I R H I P R R I R from the carboxy-terminal region of the envelope glycoprotein gp41 of HIV-1 were investigated. Using NMR and fluorescence spectroscopy on covalently labeled lipid, we detected that binding of the peptide to mixed phosphatidylcholine/phosphatidylglycerol membranes triggers the formation of domains or clusters of negatively charged phosphatidylglycerol. The peptide lowers the order of lipid headgroups and hydrocarbon chains of phosphatidylglycerol. The strength of peptide binding to the interface, structural transitions in the peptide-induced by binding, and formation of domains of negatively charged lipid in the membrane are energetically coupled. The lipid-peptide association constant reflects the changes in free energy of the lipid matrix. In particular, there is energy associated with the formation of domains of negatively charged lipid, and with structural transitions within the peptide. Using high resolution NMR

spectroscopy and circular dichroism spectropolarimetry, it was determined that the peptide adopts an ordered but flexible structure in the presence of SDS micelles and negatively charged phospholipid liposomes as well as in trifluoroethanol-water mixtures where the estimated helical content can be as high as 60%. The association of the peptide with lipid bilayers is characterized by fast exchange between different peptide conformations in the bound as well as in the free states. In the bound state the peptide assumes a set of helical conformations which are in dynamic equilibrium. Neutralization of the six arginine side chains alone does not appear to cause the peptide to adopt an ordered structure. The results favor a model of the peptide-lipid interaction where the peptide binds at the lipid/water interface. The peptide-lipid interaction is short-lived and the peptide-induced change in lipid domain organization is fast on the NMR time scale but can be detected by changes in average NMR parameters.

The translocation and activation of protein kinase C (PKC) by the plasma cell tumor-promoting alkane, pristane, were investigated. PKC appears to be of central importance for the transduction of signals affecting growth, differentiation, and function in B lymphocytes. The NMR experiments show that this branched alkane is located in the center of mixed DOPC/DOPS (4/1, wt/wt) bilayers and penetrates into the space between fatty acid chains of lipids. Pristane activates PKC in model membranes. The activation is highest at a pristane concentration of 5 mol% and decreases again at higher pristane concentration. Previously we have shown that pristane promotes the formation of nonlamellar lipid phases (Gawrisch et al., *Biochim Biophys Acta* 1991;1070:409-418). The behavior suggests that PKC activity is dependent on changes in lipid packing.

#### Significance to Biomedical Research and the Program of the Institute:

The investigation of model membranes permits qualitative and quantitative predictions for the behavior of more complex membranes found in biological tissues. The studies on ethanol binding to membranes show that ethanol binds at or near interfaces between hydrophobic and hydrophilic segments of proteins and lipids. We suggest that the altered biophysical properties of hydrophobic/hydrophilic interfaces as a result of ethanol binding may be associated with the acute and chronic effects of ethanol on membrane-bound processes. Fatty acid unsaturation is critical for function of membranes, particularly neural membranes. There is evidence that unsaturation controls activity of membrane proteins. The results of our studies of lipid-lipid interaction are the first comprehensive analysis of the influence of one to six double bonds per chain on lipid order. The results concerning protein-lipid interaction suggest that there is a link between biophysical properties of the lipid matrix and membrane protein structure and function.

#### Proposed Course:

The interaction of alcohol with proteins/lipids will be studied on selected binary lipid mixtures containing cholesterol, gangliosides, and polyunsaturated phospholipids. The investigation of lipid-lipid interactions will continue on binary and ternary lipid mixtures of neutral and charged lipids with unsaturated fatty acids. Lipid-protein interactions will be investigated on reconstituted membranes containing polyunsaturated neutral and charged lipids and rhodopsin as an example of a membrane receptor protein.

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Arnold K, Gawrisch K. Effects of fusogenic agents on membrane hydration: A deuterium nuclear magnetic resonance approach, *Methods Enzymol* 1993;220:143-157.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

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PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cerebral Energy Metabolism and Blood Flow in the Rat

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. McLaughlin Visiting Scientist LMBB, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Membrane Biochemistry and Biophysics

SECTION

Section of Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been terminated.

Publications:

Horvath I, Sandor N, Ruttner Z, McLaughlin AC. Role of nitric oxide in regulating cerebrocortical oxygen consumption and blood flow during hypercapnia, J Cereb Blood Flow Metab 1994;14:503-509.

Lyon R, McLaughlin AC. Double-quantum filtered <sup>23</sup>Na NMR studies of intracellular sodium in perfused liver, Biophys J 1994;67:369-376.

Pekar J, Sinnwell T, Ligeti L, Chesnick AS, Frank JA, McLaughlin AC. Simultaneous measurement of cerebral oxygen consumption and blood flow using <sup>17</sup>O and <sup>19</sup>F magnetic resonance imaging, J Cereb Blood Flow Metab, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00053-04 LMBB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

In Vivo 170 NMR Studies of Cerebral Oxygen Consumption and Blood Flow

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. McLaughlin Visiting Scientist LMBB, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Membrane Biochemistry and Biophysics

SECTION

Section of Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

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CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

Publications:

McLaughlin AC, Pekar J, Ligeti L, Moonen, CTW. Oxygen-17 magnetic resonance imaging of cerebral blood flow and oxygen consumption. In: LeBihan D, Rosen B, eds. Diffusion and Perfusion Magnetic Resonance Imaging. Raven Press, in press.

Pekar J, Ligeti L, Sinnwell T, Moonen CTW, Frank J, McLaughlin AC. 19F NMR imaging of cerebral blood flow with 0.4 cc resolution, J Cereb Blood Flow Metab 1994;14: 656-663.

Pekar J, Sinnwell T, Ligeti L, Chesnick AS, Frank JA, McLaughlin AC. Simultaneous measurement of cerebral oxygen consumption and blood flow using 170 and 19F magnetic resonance imaging, J Cereb Blood Flow Metab, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00056-04 LMBB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

In Vivo 31p NMR Exercise Studies of HIV-Positive Patients

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. McLaughlin Visiting Scientist LMBB, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Membrane Biochemistry and Biophysics

SECTION

Section of Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

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CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

Publications: None.





## **LABORATORY OF METABOLISM**



Annual Report of the  
Laboratory of Metabolism and Molecular Biology  
Division of Intramural Clinical and Biological Research  
National Institute on Alcohol Abuse and Alcoholism  
October 1, 1993 to September 30, 1994  
Richard L. Veech M.D., Ph.D., Chief

## Introduction

By order of the Scientific Director, NIAAA, the Laboratory of Metabolism and Molecular Biology will be abolished on September 30, 1994. During the past year, the major work of the Laboratory centered upon the effects of ethanol metabolism upon inorganic ion distributions as was mandated.

## The Relationship of Cellular Energy to Inorganic Ion Gradients

In spite of difficult circumstances, the staff continued to be remarkably productive during the past year. Several major papers resulted from our four year study upon the integration of cellular energy with the energy of inorganic ion distributions and the potential between extra and intracellular phases (Masuda T, Dobson GP, Veech RL, J Biol Chem 1990;265:20321-20334). The first addressed {VEECH1994} the effects of alteration in free intracellular  $[Mg^{2+}]$  upon many intracellular reactions, particularly those involving phosphorylated intermediates. An awareness of the importance of alterations in free  $[Mg^{2+}]$  as well as proton in determining the apparent thermodynamic constants of most important intracellular reactions led to the formulation of new IUPAC recommendations for reporting and using such data (see Alberty RA, Pure and Applied Chemistry 1994;66:1641-1666). Our report gives both the theoretical basis for these effects and a practical manner in which they may be applied. While it had been known that magnesium deficiency played a significant role in exacerbating the symptoms of alcohol withdrawal, alcoholic myopathy and hypertension, there was no understanding of how  $Mg^{2+}$  could cause such wide spread and diverse effects. We showed for the first time that administration of ethanol caused liver to loose magnesium and to decrease the free cytosolic  $[Mg^{2+}]$ . The changes in free  $[Mg^{2+}]$  were associated with changes in the energy gradients and fluxes of protons between extra and intracellular phases. Ethanol administration, increased the net charge on impermeant intracellular anions. In collaboration with electrophysiologists from the University of East Tennessee, we were able to demonstrate that the change in the gradients of protons and in the intracellular  $[Mg^{2+}]$  resulted in an increase in the potential, measured between extra and intracellular phases from -28 to -40 mV. This report was considered of enough significance to be part of a special symposium held by the NIAAA devoted entirely to magnesium in alcoholism.

The general importance of the integrated view of cellular energetics and ionic homeostasis presented above was illustrated by the application of the same principle to the well known events occurring during the transformation of normal tissues into malignant ones. Since Warburg's time, it has been thought that the intracellular phase of tumors was markedly acid, due to the accumulation of high concentrations of lactate. This premise of intracellular acidosis in tumors formed the basis of many chemotherapeutic approaches. In collaboration with the Cancer Research Campaign's NMR unit in London, {STUBBS1994} we showed that in fact the intracellular pH of tumors is the same as in normal tissue. Like normal liver metabolizing ethanol to acetate anion, tumors change the energy of their proton gradient by activating the  $Na/H^+$  exchanger, thus increasing intracellular  $[Na^+]$  and with that their intracellular  $[Ca^{2+}]$ , leading to the calcification which is the late and commonly used diagnostic hallmark of malignant transformation used in mammography. This report was of enough general interest to be the subject of an invited symposium next year.

In a fourth major report we show how thermodynamic necessity is transformed into none linear kinetic response in living tissue {KASHIWAYA1994}. We did this by showing how simple metabolites, such as ketone bodies, are able to duplicate the

metabolic effect of the hormone insulin on the metabolism of glucose. We measured all of the intermediates of glucose metabolism under four different metabolic conditions, determined the apparent equilibrium constant for all of the reactions of glycolysis (using cytosolic pH and inorganic Pi values obtained in collaboration with the NMR Unit, Department of Biochemistry, Oxford University), and determined all of the kinetic constants in the forward and backward direction for the reactions of glucose utilization. This allowed us for the first time to determine the flux control coefficients for the individual steps of glucose utilization, and define how they changed under different metabolic and hormonal conditions. What emerged was the control of the flux of glucose utilization did not reside at one "rate limiting step", but was shared by a number of reactions. Further, the degree of control, for instance the control exerted by insulin sensitive Glut 4 translocating glucose into the cell, varied depending not only upon the presence of insulin, but also upon the presence of alternative substrates. This paper was considered to be of general significance to the biochemical community and will be the subject of an editorial in *Trends in Biochemical Science* in the fall of 1994.

A number of topics under study remain unfinished and are the following:

(1) Changes in potential between extracellular phases in liver after ethanol affect the extent and direction of the Na linked uptake of amino acids. We have defined the effect of ethanol upon this process.

(2) The metabolism of ethanol fundamentally alters the gradients of  $H^+$  between mitochondria and cytosolic phases. We have developed new methods to estimate the mitochondrial and cytosolic gradients of Pi and  $H^+$ .

(3) In collaboration with the NMR group in Oxford, we have developed new methods for the estimation of free intracellular  $[Mg^{2+}]$  and have shown that the generally accepted method of measurement of  $\alpha$ - $\beta$  shift of the phosphorus resonances of ATP, a method used for the last 20 years, is inaccurate within the physiological range of  $[Mg^{2+}]$  variability.

(4) Also in collaboration with the NMR unit Oxford, we have developed new methods which for the first time elucidate the acute effects of common hormones such as insulin in changing the pH gradient and potential between cytosolic and mitochondrial phases. We have further shown how changes in the fundamental cofactor ratios rather than traditional hormonal signalling pathways account for the acute metabolic effects of insulin, such as its ability to increase the efficiency of working rat heart by 30%. This paper integrating control of mitochondrial energy transduction, redox states, and  $O_2$  consumption is of general significance in a number of areas.

(5) Finally, in collaboration with the Department of Pathology, University of Texas Medical School, Dallas, we have determined for the first time the concentration of the inorganic ions  $Na^+$ ,  $K^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Pi$ , and  $Cl^-$  in the mitochondrial, nuclear and cytosolic + endoplasmic reticular compartments of liver after treatment with ethanol and its metabolic product acetate. When combined with our space measurements, this allowed us for the first time to obtain measurements of the water content of various intracellular organelles, and thus to estimate the energy of the ion gradients between the various intracellular phases. This study is not only of significance in understanding the ability of ethanol to damage liver, but is of general significance to our understanding of many cellular processes.



Publications  
Laboratory of Metabolism  
October 1, 1993 to September 30, 1994

Kashiwaya Y, Sata K, Tsuchiya N, Thomas S, Fell DA, Veech RL, Passonneau JV. Control of glucose utilization in working perfused rat heart, J Biol Chem, in press.

Stubbs M, Rodrigues L, Howe FA, Wang J, Joeng K, Veech RL Griffiths JR. Metabolic consequences of a reversed pH gradient in rat tumors, Cancer Res 1994;54:4011-6.

Veech RL, Gates DN, Crutchfield CW, et al. Metabolic hyperpolarization of liver by ethanol. The importance of  $Mg^{2+}$  and  $H^{+}$  in determining impermanent intracellular anionic charge and energy of metabolic reactions, Alcohol Clin Exp Res, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00048-05 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Distribution in the Perfused Rat Hearts: Effect of Pi and Ethanol

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: K. Jeong Visiting Fellow LMMB, NIAAA

Others: C. Crutchfield Biological Lab Technician LMMB, NIAAA

R. Veech Chief LMMB, NIAAA

J. Wang Visiting Fellow LMMB, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Section on Metabolic Control

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00005-02 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Metabolic Events and Ion Distribution in Perfused Rat Heart

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Y. Kashiwaya Visiting Fellow LMMB, NIAAA

Others: J. Passonneau Research Chemist LMMB, NIAAA

N. Tsuchiya Visiting Fellow LMMB, NIAAA

R. Veech Chief LMMB, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Section on Metabolic Control

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

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☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00006-02 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Estimation of Cytosolic Free Phosphate in vivo

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. King Research Chemist LMMB, NIAAA

Others: J. Passonneau Research Chemist LMMB, NIAAA

R. Veech Research Chemist LMMB, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Section on Metabolic Control

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0

PROFESSIONAL:

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OTHER:

0

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☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.



**LABORATORY OF MOLECULAR AND CELLULAR NEUROBIOLOGY**





Annual Report of the  
Laboratory of Molecular and Cellular Neurobiology  
Division of Intramural Clinical and Biological Research  
National Institute on Alcohol Abuse and Alcoholism  
October 1, 1993 to September 30, 1994  
Forrest F. Weight, M.D., Chief

In recent years, great progress has been made in understanding the function of the central nervous system at the cellular and molecular level. The Laboratory of Molecular and Cellular Neurobiology was established in Fiscal Year 1992 (FY92) to utilize this increased knowledge of neurobiology to investigate the cellular and molecular basis of alcoholism and alcohol abuse. The investigations in the Laboratory are directed toward elucidating the cellular and molecular mechanisms of alcohol's acute and chronic actions, such as intoxication, tolerance and dependence, as well as pathophysiologic phenomena such as the neurotoxicity of alcohol that results in cerebral atrophy and alcohol-induced alterations in neural development that are manifested as the fetal alcohol syndrome.

Administratively, the Laboratory of Molecular and Cellular Neurobiology consists of four sections - the Section on Physiology, the Section on Pharmacology, the Section on Immunology, and the Section on Molecular Neuroscience. The Sections on Physiology, Pharmacology, and Immunology were previously established research programs when the Laboratory of Molecular and Cellular Neurobiology was formed in FY92; the Section on Molecular Neuroscience was newly established in FY92. Dr. Forrest Weight is Chief, Section on Physiology, and Acting Chief, Section on Molecular Neuroscience. The Chief, Section on Pharmacology, Dr. George Kunos, resigned in 1992 to become Chairman, Department of Pharmacology, Virginia Commonwealth University, and the Chief, Section on Immunology, Dr. Randall Kincaid, resigned in 1993 to become Director, Cell Biology, Human Genome Sciences, Inc. Due to a hiring freeze and budget cuts in both FY93 and FY94, the vacancies created by the resignation of the staff in the Sections on Pharmacology and Immunology have not been filled; consequently, there will be no report on the research activities of those sections for FY94. The research activities of the Section on Physiology and the Section on Molecular Neuroscience are discussed in more detail below.

The Section on Physiology investigated the neuronal actions of alcohol and provided further evidence demonstrating that neurotransmitter receptors are molecular sites for alcohol action in the nervous system. This evidence is a major advance in alcohol research, as it had been thought for over 90 years that the neural effects of alcohol are due to nonspecific actions on membrane lipids. Since neurotransmitter receptors mediate communication between neurons at synapses, the demonstration that alcohol affects the function of these receptors suggests that these actions may underlie the behavioral effects of alcohol.

The Section on Molecular Neuroscience used molecular biological approaches to investigate the molecular basis of alcohol action on neurotransmitter receptors. Initial studies of the Section have provided evidence that ethanol sensitivity of some types of neurotransmitter receptors can be determined by the subunit composition of the receptor. In addition, studies are in progress on the molecular regulation of alcohol sensitivity. These studies hold the promise that such molecular biological approaches will advance our knowledge of the molecular basis of alcohol actions in the nervous system.

#### Section on Physiology

The research program of the Section on Physiology is directed toward elucidating the cellular mechanisms of alcohol actions in the nervous system. The behavioral effects of alcohol are well known; however, the mechanisms by which alcohol produces those effects have not been established. The investigations in the Section use primarily neurophysiological methods such as patch-clamp and voltage-clamp techniques to study the actions of alcohol on mammalian neurons and neuronal membrane ion channels.

Experiments on neuronal voltage-gated ion channels indicate that these channels, which underlie the intrinsic electrical excitability of neurons, are relatively insensitive to pharmacologic concentrations of ethanol (5-100 mM). By contrast, a number of neuronal neurotransmitter-gated ion channels have been found to be sensitive to ethanol in pharmacologic concentrations. This type of neurotransmitter receptor mediates excitatory and inhibitory synaptic transmission between neurons in the central nervous system. The effects of ethanol on these ion channels are discussed in more detail below.

#### A. Excitatory Amino Acid-Activated Channels

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). Glutamate activates at least three types of neurotransmitter-gated ion channels, designated by their responses to the agonists: N-methyl-D-aspartate (NMDA), kainate, and quisqualate (or AMPA).

NMDA-gated channels mediate a slow synaptic excitation and are thought to be involved in excitatory neural phenomena, synaptic plasticity, cognitive function, and certain types of motor control. In hippocampal neurons, ethanol inhibits NMDA-activated current in a concentration-dependent manner over the concentration range 5-50 mM, a range that produces intoxication. The average inhibition by 50 mM ethanol is 45-60% and the concentration that produces 50% inhibition ( $IC_{50}$ ) is ~30 mM. The potency of several short chain alcohols for inhibiting NMDA-activated current is related to their intoxicating potency, suggesting that alcohol-induced inhibition of NMDA channel function may contribute to the neural and cognitive impairments associated with intoxication. Investigations on the mechanism involved in the inhibition of NMDA current by ethanol indicate that ethanol does not inhibit NMDA current by voltage-dependent block of the ion channel, by altering the ion selectivity of the channel or by interaction with several regulatory sites on the channel. Single channel experiments suggest that ethanol inhibits NMDA-activated current by altering channel gating. A series of straight chain alcohols exhibits a cutoff in potency for inhibiting NMDA receptors, suggesting that alcohols inhibit the function of NMDA-gated ion channels by interacting with a hydrophobic pocket on the channel protein. In addition, this cutoff is similar to the cutoff in the potency of these alcohols for producing intoxication, suggesting that the cutoff for alcohol inhibition of NMDA receptors may underlie the cutoff for alcohol intoxication. This implication supports the hypothesis that alcohol inhibition of NMDA receptors may be involved in alcohol intoxication.

Kainate and quisqualate channels mediate fast transmission at the majority of excitatory synapses in the CNS. In concentrations  $\leq 50$  mM, ethanol has a relatively small effect on the current activated by kainate or quisqualate. In concentrations greater than 50 mM, ethanol produces an increasing concentration-dependent inhibition of these currents, with 200 mM ethanol inhibiting these currents by about 45%. Blood-ethanol concentrations greater than 50 mM are associated with signs of general anesthesia, suggesting that the inhibition of kainate and quisqualate channel function may contribute to the general anesthetic effects of ethanol. This hypothesis is supported by observations that the general anesthetic agents, trichloroethanol (the active metabolite of chloral hydrate), barbiturates, and volatile anesthetics, all inhibit kainate- and quisqualate-activated currents in a pharmacologic concentration range.

#### B. Inhibitory Amino Acid-Activated Channels

The major inhibitory neurotransmitter in brain is gamma-aminobutyric acid (GABA). Ethanol has been found to have different effects on GABA-gated ion channels (GABA<sub>A</sub> receptors) in different preparations. In cultured mouse cortical and hippocampal neurons, 1-40 mM ethanol has been found to produce a concentration-dependent potentiation of GABA-activated current in some, but not all, neurons tested. By contrast, ethanol concentrations from 10-100 mM have no significant effect on GABA-activated current in adult rat dorsal root ganglion (DRG) neurons. Benzodiazepines selectively potentiate GABA<sub>A</sub> current, suggesting that the potentiation of this current by ethanol may contribute to the anxiolytic actions

of ethanol.

#### C. Serotonin-Activated Channels

Recent studies indicate that the neurotransmitter serotonin (5-HT) can activate a ligand-gated ion channel, which has been designated the 5-HT<sub>3</sub> channel. In NCB-20 cells and nodose neurons, ethanol can potentiate 5-HT<sub>3</sub> current in a concentration-dependent manner over the concentration range 25-100 mM. Maximal potentiation of 59% is observed with an ethanol concentration of 100 mM. Potentiation by ethanol decreases with increasing serotonin concentration, suggesting that ethanol may increase the potency of serotonin action. Behavioral experiments suggest that the interaction of ethanol with 5-HT<sub>3</sub> receptors may be related to recognition of ethanol action. Potentiation of 5-HT<sub>3</sub> receptor-mediated ion current by a series of straight chain alcohols exhibits a distinct cutoff for alcohols with six or more carbon atoms, suggesting that alcohols potentiate 5-HT<sub>3</sub> receptor-mediated ion current by interacting directly with a hydrophobic pocket on the receptor protein.

Recent behavioral studies have also implicated 5-HT<sub>3</sub> receptors in the reinforcing properties of several drugs of abuse, including cocaine. In investigations on the interaction of cocaine with 5-HT<sub>3</sub> receptors, cocaine competitively inhibits serotonin activation of 5-HT<sub>3</sub> receptors with a pA<sub>2</sub> value of 5.4 and an apparent K<sub>d</sub> of 3.8  $\mu$ M. This effect occurs at pharmacologically relevant concentrations of cocaine, suggesting that this action may contribute to the psychoactive effects of cocaine.

#### D. ATP-Activated Channels

Adenosine 5'-triphosphate (ATP) has recently been reported to function, extracellularly, as a neurotransmitter and to activate ligand-gated ion channels in vertebrate neurons. There appear to be several types of ATP-gated ion channels in mammalian neurons, based on activation and desensitization kinetics, and pharmacologic sensitivity. Ethanol inhibits the function of one type of ATP-gated channel over the concentration range 6-250 mM. The average inhibition of current activated by 1  $\mu$ M ATP by 100 mM ethanol is 64%, and the IC<sub>50</sub> is 68 mM. Methanol is less potent and 1-propanol is more potent in inhibiting the ATP-activated current; however, 1-butanol and 1-pentanol are without effect on this current, suggesting that alcohols with three carbons or less affect the function of this receptor by interacting directly with a small hydrophobic pocket on the receptor protein, rather than by an action on membrane lipids.

#### E. Summary and Conclusions

Taken together, the preceding studies indicate that neurotransmitter-gated ion channels are molecular sites of ethanol action in the nervous system. In view of the important role that neurotransmitter-gated ion channels play in CNS information processing, the observations that ethanol can affect the function of these receptors suggests that these actions may contribute to the behavioral effects of ethanol.

#### Section on Molecular Neuroscience

The research activities of the Section on Molecular Neuroscience are directed toward understanding actions of alcohol in the nervous system at the molecular level. The Section uses a combination of molecular biological and electrophysiological research methods to address these questions. In the initial studies carried out by the Section, the effects of ethanol have been studied on the physiology and pharmacology of recombinant neurotransmitter receptors using *Xenopus* oocytes as an expression system. Those studies are summarized briefly below.

#### A. Differential Ethanol Sensitivity of Recombinant NMDA Receptor Subunits

Although ethanol has been found to inhibit NMDA receptor-mediated responses in



a number of regions of the nervous system, the sensitivity to ethanol is different in different types of neurons. Recent cloning studies have revealed a molecular diversity of NMDA receptors and a differential distribution of different subunits throughout the brain, raising the question of whether differences in NMDA receptor subunit composition might be responsible for the differences in NMDA receptor sensitivity to ethanol in different types of neurons.

We have found that recombinant NMDA receptor subunits expressed in *Xenopus* oocytes are differentially sensitive to ethanol. The heteromeric subunit combinations epsilon1/zeta1 and epsilon2/zeta1 are significantly inhibited by 50 mM ethanol, whereas the heteromeric combination epsilon3/zeta1 and the homomeric zeta1 are not significantly affected by this concentration of ethanol. In addition, there are differences in the ethanol concentration-response curves for different subunit combinations. The observations are consistent with the idea that the NMDA receptor subunit composition may contribute to differences in ethanol sensitivity observed in different types of neurons.

#### B. Ethanol Potentiation of 5-HT<sub>1</sub> Receptor-Mediated Ion Current in *Xenopus* oocytes

Studies in the Physiology Section of this Laboratory have shown that ethanol can potentiate 5-HT<sub>1</sub> receptor-mediated ion current in neurons and neural cell lines; however, 5-HT<sub>1</sub> current is insensitive to ethanol in approximately 15% to 25% of these cells. Since one subunit of the 5-HT<sub>1</sub> receptor has been cloned, we expressed this subunit in *Xenopus* oocytes to determine whether the expressed homomeric receptor subunit is sensitive to ethanol (as noted above, homomeric expression of the NMDA receptor subunit, zeta1, is not sensitive to ethanol, whereas heteromeric expression can be). We found that for the recombinant homomeric 5-HT<sub>1</sub> receptor, ethanol consistently potentiates the current activated by 5-HT in all of the cells studied (n=60). Since this clone of the 5-HT<sub>1</sub> receptor has protein kinase A (PKA), tyrosine kinase and casein kinase phosphorylation sites, experiments are currently in progress, using molecular biological methods such as site-directed mutagenesis, to determine whether these phosphorylation sites can regulate the sensitivity of 5-HT<sub>1</sub> receptors to ethanol.

#### C. Ethanol Does Not Affect GABA-Activated Current in *Xenopus* Oocytes Expressing Long-Sleep Mouse Brain mRNA or Recombinant alpha1beta1gamma2L Subunits

Another laboratory had reported that ethanol can potentiate GABA-activated current in *Xenopus* oocytes expressing either long-sleep (LS) mouse brain mRNA or GABA<sub>A</sub> alpha1beta1gamma2L subunit recombinant cRNA. However, despite over two years of research attempting to repeat these observations, we have been unable to find any effect of ethanol in *Xenopus* oocytes expressing either LS mouse brain mRNA or GABA<sub>A</sub> alpha1beta1gamma2L subunit recombinant cRNA. Our inability to confirm these previous reports suggests that the molecular determinants of GABA<sub>A</sub> receptor sensitivity to ethanol are not resolved.

#### D. Summary and Conclusions

The initial studies of the Section on Molecular Neuroscience suggest that the application of molecular biological approaches to the study of alcohol interactions with biological systems will advance our knowledge of the molecular basis of alcohol actions in the nervous system.

Publications  
Laboratory of Molecular and Cellular Neurobiology  
October 1, 1993 to September 30, 1994

Aguayo LG, Grossie J. Dopamine inhibits a sustained calcium current through activation of alpha adrenergic receptors and a GTP-binding protein in adult rat sympathetic neurons, *J Pharmacol Exp Ther* 1994;269:503-8.

Aguayo LG, Pancetti FC. Ethanol modulation of the GABA<sub>A</sub>- and glycine-activated Cl<sup>-</sup> current in cultured mouse neurons, *J Pharmacol Exp Ther*, in press.

Aime C, Bristol LA, Berrih S, Durum S, Takacs L. Detection of IL-1 mRNA expression in the normal and neoplastic human thymus by in situ hybridization and PCR: Correlation with late T-cell maturation in normal versus neoplastic thymus, *Thymus* 1993;22:45-8.

Gotlieb WH, Bristol LA, Weissman AM, Durum SK, Takacs L. Upregulation of T-cell receptor gamma chain transcription by interleukin-2, *Cell Immunol* 1993;151:345-55.

Hager G, Dodt H-U, Zieglgansberger W, Liesi P. Novel forms of neuronal migration in the rat cerebellum, *J Neurosci Res*, in press.

Li C, Aguayo L, Peoples RW, Weight FF. Ethanol inhibits a neuronal ATP-gated ion channel, *Mol Pharmacol* 1993;44:871-5.

Li C, Peoples RW, Li Z, Weight FF. Zn<sup>2+</sup> potentiates excitatory action of ATP on mammalian neurons, *Proc Natl Acad Sci USA* 1993;90:8264-7.

Li C, Peoples RW, Weight FF. Alcohol action on a neuronal membrane receptor: Evidence for a direct interaction with the receptor protein, *Proc Natl Acad Sci USA*, in press.

Liesi P, Hager G, Seppala I, Zieglgansberger W. Domain specific antibodies against B2 chain of laminin inhibit neuronal migration in the neonatal rat cerebellum, *J Neurosci Res*, in press.

Lovinger DM, Peoples RW. Actions of alcohols and other sedative/hypnotic compounds on cation channels associated with glutamate and 5-HT<sub>2</sub> receptors. In: Alling C, et al., eds. *Alcohol, Cell Membranes and Signal Transduction in Brain*. New York: Plenum Press, 1993;157-67.

Masood K, Wu C, Brauneis U, Weight FF. Differential ethanol sensitivity of recombinant N-Methyl-D-aspartate receptor subunits, *Mol Pharmacol* 1994;45:324-9.

Muramatsu T, Kincaid RL. Molecular cloning of a full-length cDNA encoding the catalytic subunit of human calmodulin-dependent protein phosphatase (Calcineurin  $\alpha$ ), *Biochim Biophys Acta* 1993;1178:117-20.

Paliogianni F, Kincaid RL, Boumpas DT. Prostaglandin E<sub>2</sub> and other cyclic AMP elevating agents inhibit interleukin-2 gene transcription by counteracting calcineurin-dependent pathways, *J Exp Med* 1993;178:1813-7.

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Polli JW, Kincaid RL. Expression of a 63 kDa calmodulin-dependent phosphodiesterase isoform (PDE1B1) correlates with brain regions having extensive dopaminergic innervation, *J Neurosci* 1994;14:1251-61.



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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00404-07 LMCN

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of Calcium- and Phosphorylation-Regulated Signalling Pathways

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. Kincaid Special Volunteer LMCN, NIAAA

Others: A. Gubin Visiting Fellow LMCN, NIAAA

C. Marietta Research Physiologist LMCN, NIAAA

S. Matsushita Visiting Fellow LMCN, NIAAA

COOPERATING UNITS (if any)

Penn State U (M. Billingsley); FDA (M. Moos); Harvard U (K. Kosik); Duke U (A. Means); Merck Res. Lab. (S. O'Keefe, M. Tocci)

LAB/BRANCH

Laboratory of Molecular and Cellular Neurobiology

SECTION

Section on Immunology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been terminated due to the departure of the Principal Investigator.

References:

Muramatsu T, Kincaid RL. Molecular cloning of a full-length cDNA encoding the catalytic subunit of human calmodulin-dependent protein phosphatase (calcineurin Aalpha), *Biochim Biophys Acta* 1993;1178:117-20.

Paliogianni F, Kincaid RL, Boumpos DT. Prostaglandin E1 and other cyclic AMP elevating agents inhibit interleukin-2 gene transcription by counteracting calcineurin-dependent pathways, *J Exp Med* 1993;1813-17.

Polli JW, Kincaid RL. Expression of a 63 kDa calmodulin-dependent phosphodiesterase isoform (PDE1B1) correlates with brain regions having extensive dopaminergic innervation, *J Neurosci* 1994;14:1251-61.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00007-02 LMCN

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Neurobiology and Alcohol

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	F. Weight	Chief	LMCN, NIAAA
Others:	E. Akinshola	IRTA Fellow	LMCN, NIAAA
	U. Brauneis	Senior NRC Associate	LMCN, NIAAA
	E. Lazar-Wesley	Senior Staff Fellow	LMCN, NIAAA
	P. Liesi	Visiting Scientist	LMCN, NIAAA
	N. Lobaugh	NRC Fellow	LMCN, NIAAA
	K. Masood	Senior Staff Fellow	LMCN, NIAAA
	M. Oz	Visiting Fellow	LMCN, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Molecular and Cellular Neurobiology

SECTION

Section on Molecular Neuroscience

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

9.5

PROFESSIONAL:

9.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project studied the molecular basis of alcohol actions in the nervous system. The recombinant N-methyl-D-aspartate (NMDA) receptor subunit  $\zeta 1$  and the heteromeric subunit combinations  $\epsilon 1/\zeta 1$ ,  $\epsilon 2/\zeta 1$ , and  $\epsilon 3/\zeta 1$  were expressed in *Xenopus* oocytes, and their sensitivity to ethanol was investigated using the voltage-clamp technique. NMDA-activated currents in oocytes expressing  $\epsilon 1/\zeta 1$  or  $\epsilon 2/\zeta 1$  were significantly inhibited by 50 mM ethanol, whereas NMDA-activated currents associated with  $\zeta 1$  or  $\epsilon 3/\zeta 1$  combination were not significantly affected by 50 mM ethanol. Ethanol decreased the maximal amplitude ( $E_{max}$ ) of the concentration-response curve for NMDA-activated current, without significantly affecting the  $EC_{50}$ . The percentage inhibition by ethanol was not significantly different for current activated by NMDA concentrations from 10 to 250  $\mu M$ . NMDA current associated with  $\epsilon 1/\zeta 1$  was increasingly inhibited by increasing concentrations of ethanol from 25 to 100 mM, whereas 25 mM ethanol elicited near maximal inhibition of NMDA current associated with  $\epsilon 2/\zeta 1$ . NMDA current associated with  $\epsilon 3/\zeta 1$ , on the other hand, was only inhibited by 100 mM ethanol, and NMDA current associated with  $\zeta 1$  was not significantly affected by ethanol concentrations up to 100 mM. The effect of ethanol was also studied on cloned 5-HT<sub>3</sub> receptors expressed in *Xenopus* oocytes. Ethanol concentrations from 20 to 40 mM potentiated, in a concentration-dependent manner, currents activated by 250 or 500 nM 5-HT ( $EC_{50}$ =126 mM). On the other hand, we did not find ethanol sensitivity of GABA-A receptor-mediated ion currents in *Xenopus* oocytes expressing long-sleep mouse brain mRNA or recombinant GABA-A receptors containing  $\alpha 1\beta 1\gamma 2L$  subunits. The observations suggest that molecular biological techniques will be useful for elucidating the molecular determinants of alcohol actions in the nervous system.

Project Description:Investigators:

F. Weight	Chief	LMCN, NIAAA
E. Akinshola	IRTA Fellow	LMCN, NIAAA
U. Brauneis	Senior NRC Associate	LMCN, NIAAA
E. Lazar-Wesley	Senior Staff Fellow	LMCN, NIAAA
P. Liesi	Visiting Scientist	LMCN, NIAAA
N. Lobaugh	NRC Fellow	LMCN, NIAAA
K. Masood	Senior Staff Fellow	LMCN, NIAAA
M. Oz	Visiting Fellow	LMCN, NIAAA
C. Wu	Visiting Associate	LMCN, NIAAA
L. Zhang	Visiting Associate	LMCN, NIAAA

Objectives:

The objective of this project was to elucidate the molecular mechanisms of alcohol actions in the nervous system.

Methods Employed:

This project used predominantly molecular biological and electrophysiological research methods. The techniques used are briefly described as follows.

Molecular Biology

For studies of recombinant receptors, cDNA clones were provided by Drs. M. Mishina (NMDA), S. Heinemann (GluR), D. Burt (GABA<sub>A</sub>), and D. Julius (5-HT<sub>3</sub>). cRNAs were synthesized *in vitro* from linearized templates of the corresponding cDNAs. For studies of brain mRNA, polyadenylated RNA was prepared from whole brain using the Fast Track isolation kit (Invitrogen, San Diego, CA).

Oocyte Injection

*Xenopus laevis* oocytes were isolated using 0.2% collagenase A to remove connective tissue and follicular membranes. The oocytes were injected with cRNA or mRNA and incubated for two to three days in modified Barth's saline solution before being used for electrophysiological recording.

Electrophysiological Recording

After sufficient incubation for receptor expression (usually two to three days), oocytes were placed in a recording chamber (vol. ~100  $\mu$ L) and superfused with frog Ringer solution. Standard two-electrode voltage-clamp was used to record membrane ion currents; membrane holding potential was usually -70 mV. Agonists and drugs were applied to the oocytes by a fast superfusion system, using a macropipette.

Data Acquisition

The output of the voltage-clamp amplifier was recorded on a rectilinear pen recorder (Gould 2400) and was digitized and stored on magnetic media for subsequent analysis using a Compaq 386/20e microcomputer.

Major Findings:Differential Ethanol Sensitivity of Recombinant NMDA Receptor Subunits

The recombinant N-methyl-D-aspartate (NMDA) receptor subunit zeta1, and the heteromeric subunit combinations epsilon1/zeta1, epsilon2/zeta1, and epsilon3/zeta1 were expressed in *Xenopus* oocytes and their sensitivity to ethanol was investigated using the two-electrode voltage-clamp technique. NMDA-activated currents in oocytes expressing subunit combinations epsilon1/zeta1 or epsilon2/zeta1 were significantly inhibited by 50 mM ethanol, whereas NMDA-activated currents associated with the homomeric expression of zeta1 or the heteromeric epsilon3/zeta1 combination were not significantly affected by 50 mM



ethanol. Ethanol decreased the maximal amplitude ( $E_{max}$ ) of the concentration-response curve for NMDA-activated current, without significantly affecting the  $EC_{50}$ . The percentage inhibition by ethanol was not significantly different, regardless of the amplitude of current activated by NMDA concentrations from 10 to 250  $\mu$ M. Different NMDA receptor subunits and subunit combinations exhibited differences in the concentration-response curves for ethanol. NMDA-activated current associated with the epsilon1/zeta subunit combination was increasingly inhibited by increasing concentrations of ethanol from 25 to 100 mM, whereas 25 mM ethanol elicited near maximal inhibition of NMDA-activated current associated with the epsilon2/zeta subunits - viz. the inhibition by 50 or 100 mM ethanol was not significantly different. NMDA-activated current associated with the epsilon3/zeta subunit combination, on the other hand, was only significantly inhibited by 100 mM ethanol, and NMDA-activated current associated with the homomeric zeta subunit was not significantly affected by ethanol concentrations  $\leq$  100 mM. Since NMDA receptor subunits are differentially distributed throughout the brain, the observations suggest that the differential sensitivity of NMDA receptor subunits to ethanol may contribute to the differences in ethanol sensitivity observed in different types of neurons.

Ethanol Potentiation of 5-HT<sub>3</sub> Receptor-Mediated Ion Current in *Xenopus* Oocytes  
The effect of ethanol on 5-HT<sub>3</sub> receptor-mediated current was studied in *Xenopus* oocytes voltage-clamped at -70 mV that had been previously microinjected with RNA transcripts from the cloned 5-HT<sub>3</sub> receptor (Science 1991;254:432). In these cells, 5-HT application activated a fast inward current. Ethanol concentrations from 20 to 400 mM potentiated, in a concentration-dependent manner, currents activated by 250 nM or 500 nM 5-HT. The threshold for significant potentiation was 40 mM ethanol (One Factor ANOVA;  $P < 0.01$ ) and the  $EC_{50}$  was 126 mM. The potentiation was consistently observed in all 60 oocytes tested, in which 250 nM or 500 nM 5-HT was applied. With these concentrations of 5-HT, the potentiation by 80 mM ethanol was 45% and 35%, respectively, whereas with concentrations of 5-HT  $\geq$  1  $\mu$ M, no potentiation of 5-HT current was observed. The observations indicate that intoxicating concentrations of ethanol can potentiate 5-HT<sub>3</sub> receptor-mediated responses in *Xenopus* oocytes injected with a single subunit of the 5-HT<sub>3</sub> receptor. Since the pattern of this potentiation is similar to previous results from this laboratory using NCB20 cells (Neurosci Lett 1991;122:57) and nodose neurons (Mol Pharmacol 1991;40:263), further molecular biological study of the mechanism by which ethanol potentiates 5-HT<sub>3</sub> receptor-mediated responses can be carried out using this expression system.

Ethanol Does Not Affect GABA-Activated Current in *Xenopus* Oocytes Expressing Long-Sleep Mouse Brain mRNA or Recombinant  $\alpha$ 1 $\beta$ 1gamma2L Subunits

In studies on neurons, both ethanol-sensitivity and ethanol-insensitivity of GABA<sub>A</sub> receptors have been reported (cf. Intl Rev Neurobiol 1992;33:289-348). One possible reason for such differences in ethanol sensitivity is differences in the molecular structure of GABA<sub>A</sub> receptors in different types of neurons. In this regard, Wafford et al. (Neuron 1991;7:27-33) have proposed that the gamma2L subunit is required for ethanol sensitivity of GABA<sub>A</sub> receptors. This conclusion was based primarily on two observations using two-electrode voltage-clamp to study GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes: (1) in oocytes expressing long-sleep (LS) mouse brain mRNA, ethanol (20 mM) enhancement of current activated by GABA (30  $\mu$ M) was prevented by antisense oligonucleotides to the gamma2L subunit; and (2) when either recombinant mouse  $\alpha$ 1 $\beta$ 1gamma2S or  $\alpha$ 1 $\beta$ 1gamma2L subunit combinations were expressed, ethanol (20 mM) potentiated the current activated by GABA (30  $\mu$ M) only in the combination containing the gamma2L subunit. In order to study this ethanol enhancement in more detail, we used the two-electrode voltage-clamp technique to study LS mouse brain mRNA expressed in *Xenopus* oocytes. However, we were unable to find any effect of ethanol on the GABA-activated current with ethanol concentrations from 1 to 80 mM and GABA concentrations from 1 to 60  $\mu$ M. Since we were unable to study ethanol enhancement of these GABA currents, we expressed rat  $\alpha$ 1 $\beta$ 1gamma2L cRNA in *Xenopus* oocytes (rat subunits were used initially because we could not obtain the mouse

$\beta 1$  cDNA, because the sequence had not been published). However, we were unable to find any effect of ethanol on the GABA-activated current of the rat recombinant receptors with 20 mM ethanol and GABA concentrations from 1 to 100  $\mu$ M. When the mouse cDNAs became available, we also tested the effect of ethanol on GABA<sub>A</sub> receptors containing mouse  $\alpha 1\beta 1\gamma 2L$  subunits. However, as with the rat recombinant receptors, we were unable to find any effect of ethanol on the GABA-activated current of the mouse recombinant receptors with ethanol concentrations from 5 to 100 mM and GABA concentrations from 0.3 to 100  $\mu$ M. The reason for the difference between our observations and those of Wafford et al. (1991) is not understood. It should be noted, however, that in our experiments on mouse recombinant receptors, 0.1  $\mu$ M diazepam potentiated current activated by 5  $\mu$ M GABA by 182%, indicating that the gamma subunit was expressed. In addition, 100  $\mu$ M pentobarbital potentiated the current activated by 5  $\mu$ M GABA by 582% and 5 mM trichloroethanol potentiated the current activated by 1  $\mu$ M GABA by 1,900%, indicating that the mouse recombinant receptors can be allosterically modulated by both barbiturates and alcohols.

#### Significance to Biomedical Research and the Program of the Institute:

The molecular basis of alcohol actions in the nervous system is poorly understood. This project suggests that the combined use of molecular biological and electrophysiological techniques will be useful for elucidating the molecular determinants of alcohol actions in the nervous system.

#### Proposed Course:

The molecular determinants of alcohol sensitivity will continue to be studied. In addition, molecular biological techniques such as chimeras and site-directed mutagenesis will be used in an attempt to determine the molecular sites of alcohol interaction with membrane receptors, and whether regulatory mechanisms such as phosphorylation or glycosylation are involved in alcohol effects on receptor function.

#### Publications:

Aime C, Bristol LA, Berrih S, Durum S, Takacs L. Detection of IL-1 mRNA expression in the normal and neoplastic human thymus by in situ hybridization and PCR: Correlation with late T-cell maturation in normal versus neoplastic thymus, Thymus 1993;22:45-48.

Gotlieb WH, Bristol LA, Weissman AM, Durum SK, Takacs L. Upregulation of T-cell receptor gamma chain transcription by interleukin-2, Cell Immunol 1993;151:345-55.

Hager G, Dodt H-U, Zieglgansberger W, Liesi P. Novel forms of neuronal migration in the rat cerebellum, J Neurosci Res, in press.

Liesi P, Hager G, Seppala I, Zieglgansberger W. Domain specific antibodies against B2 chain of laminin inhibit neuronal migration in the neonatal rat cerebellum, J Neurosci Res, in press.

Masood K, Wu C, Brauneis U, Weight FF. Differential ethanol sensitivity of recombinant N-Methyl-D-aspartate receptor subunits, Mol Pharmacol 1994;45:324-329.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00479-11 LMCN

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synaptic Mechanisms and Alcohol Actions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	F. Weight	Chief	LMCN, NIAAA
Others:	P. Fan	Visiting Fellow	LMCN, NIAAA
	C. Li	IRTA Fellow	LMCN, NIAAA
	R. Peoples	IRTA Fellow	LMCN, NIAAA
	A. Ravindran	Visiting Scientist	LMCN, NIAAA
	J. Wright	Senior Staff Fellow	LMCN, NIAAA
	O. Yu	NRSA Fellow	LMCN, NIAAA
	J. Zhai	Visiting Associate	LMCN, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Molecular and Cellular Neurobiology

SECTION

Section on Physiology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

7.5

PROFESSIONAL:

7.5

OTHER:

0.0

CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project investigated the mechanism of alcohol effects on neurotransmitter receptors using the patch-clamp technique. Previous experiments showed that ethanol can potentiate the function of 5-HT<sub>3</sub> receptor-ion channels, and inhibit the function of ATP- and NMDA-gated ion channels. Recent experiments investigated the effect of different alcohols on the function of these ligand-gated ion channels. The inhibition of ATP-activated ion current by a series of straight chain alcohols exhibits a distinct cutoff effect. For alcohols with a molecular volume of less than 42.2 ml/mol, potency for inhibiting ATP-activated current is correlated with lipid solubility (1-propanol, trifluoroethanol, monochloroethanol, ethanol, methanol). However, despite increased lipid solubility, alcohols with a molecular volume of greater than 46.1 ml/mol (1-butanol, 1-pentanol, trichloroethanol, and dichloroethanol) are without effect on the ATP-activated current. The potentiation of 5-HT<sub>3</sub> receptor-mediated current by 1-propanol, 1-butanol, and 1-pentanol is concentration-dependent, with EC<sub>50</sub> values of 34.2, 2.7, and 1.4 mM, respectively. However, 1-hexanol (0.5 to 50 mM) and 1-octanol (0.05 to 5 mM) do not affect the amplitude of 5-HT<sub>3</sub> receptor-mediated current. The inhibition of NMDA-activated ion current by a series of straight chain alcohols also exhibits a distinct cutoff. Although potency for inhibition of NMDA receptors increases exponentially for alcohols with one to five carbon atoms, as the number of carbon atoms increases from six to eight, potency for inhibition of NMDA receptors levels off and then decreases. The cutoffs for ATP, 5-HT<sub>3</sub>, and NMDA receptors suggest that the alcohols affect the function of these neurotransmitter receptors by interacting directly with a circumscribed hydrophobic pocket on the receptor protein. The difference in the cutoffs for different receptors suggests that the size of the hydrophobic pocket is different on different types of receptors.



Project Description:Investigators:

P. Fan	Visiting Fellow	LMCN, NIAAA
C. Li	IRTA Fellow	LMCN, NIAAA
R. Peoples	IRTA Fellow	LMCN, NIAAA
A. Ravindran	Visiting Scientist	LMCN, NIAAA
F. Weight	Chief	LMCN, NIAAA
J. Wright	Senior Staff Fellow	LMCN, NIAAA
O. Yu	NRSA Fellow	LMCN, NIAAA
J. Zhai	Visiting Associate	LMCN, NIAAA

Objectives:

The objective of this project is to elucidate the cellular mechanisms of alcohol actions in the nervous system.

Methods Employed:

The actions of alcohols on neurotransmitter receptors were investigated in dissociated and cultured neurons using electrophysiological techniques. The techniques used are briefly described below.

Dissociated Neurons

Neurons were dissociated from the nodose, superior cervical and dorsal root ganglia, and from the hippocampal region of the central nervous system of adult rats or bullfrogs using mechanical and enzymatic treatment (Ikeda et al., J Neurophysiol 1986;55:527). Electrophysiological recordings were obtained from these neurons within a period of ten hours after dissociation.

Cultured Neurons

Hippocampal, cortical and spinal cord neurons were dissociated from 16- to 17-day mouse or rat embryos and grown in tissue culture using the method of Forsythe and Westbrook (J Physiol 1988;396:515). Electrophysiological experiments were performed two to four weeks after plating.

Electrophysiological Recording

In many experiments, membrane ion currents were recorded using the whole-cell patch-clamp method (cf. Ikeda et al., 1986). Receptor agonists and various pharmacologic agents were administered from large-bore micropipettes (>40  $\mu$ m tip diameter) placed near the cell soma. In experiments recording single channel currents, outside-out tear-off patch-clamp recording was used (cf. Hamill et al., Pflügers Arch 1981;391:85).

Data Acquisition

The output of the patch-clamp amplifier was digitized and stored on magnetic media for subsequent analysis using a Compaq 386/20e microcomputer. The microcomputer was also used to generate various patterns of voltage commands.

Major Findings:Cutoff in Alcohol Inhibition of ATP-Gated Ion Channel Function

Previous studies in this laboratory have shown that ethanol, in a pharmacologic concentration range, can inhibit the function of ATP-gated ion channels (Mol Pharmacol 1993;44:871-875). In recent experiments, we investigated the effect of several different alcohols on the current activated by 2.5  $\mu$ M ATP. Ethanol, in a concentration of 100 mM, markedly decreased the amplitude of ATP-activated current. On average, 100 mM ethanol reduced the amplitude of ATP-activated current by 45 $\pm$ 3% (n=8). Methanol (200 mM) and 1-propanol (50 mM) also markedly decreased the amplitude of ATP-activated current. On average, the amplitude of ATP-activated current was decreased 34 $\pm$ 3% (n=7) by 200 mM methanol and 57 $\pm$ 5%

(n=7) by 50 mM 1-propanol. The inhibition of ATP-activated current by methanol, ethanol, and 1-propanol was concentration-dependent, and the concentrations that produced 50% inhibition ( $IC_{50}$ ) were 298 mM, 110 mM, and 47 mM, respectively. By contrast, 1-butanol did not significantly affect the amplitude of ATP-activated current at any concentration between 2 and 30 mM (ANOVA;  $P>0.1$ ; n=5-8), and 1-pentanol did not significantly affect the amplitude of ATP-activated current at any concentration between 1.5 and 10 mM (ANOVA;  $P>0.1$ ; n=5-8). The highest concentrations of 1-butanol and 1-pentanol tested would produce membrane alcohol concentrations equivalent to or higher than that produced by 500 mM ethanol. The effects of several halogenated alcohols on ATP-activated current were also tested. Monochloroethanol and trifluoroethanol inhibited ATP-activated current in a concentration-dependent manner, with  $IC_{50}$  values of 94 mM and 48 mM, respectively. However, dichloroethanol did not significantly affect the amplitude of ATP-activated current at any concentration between 2 and 50 mM, and trichloroethanol did not significantly affect the amplitude of ATP-activated current at any concentration between 0.3 and 5 mM (ANOVA;  $P>0.1$ ; n=5-8). The highest concentrations of dichloroethanol and trichloroethanol tested would produce membrane alcohol concentrations equivalent to or higher than that produced by 500 mM ethanol. Analysis of these data indicates that for alcohols with a molecular volume  $\leq 42.2$  ml/mol, potency for inhibiting ATP-activated current is correlated with molecular volume (methanol < ethanol < monochloroethanol < trifluoroethanol = 1-propanol), and there is a significant linear relationship between these two measures (linear regression analysis of variance;  $P<0.001$ ). However, despite increased lipid solubility, alcohols with a molecular volume  $\geq 46.2$  ml/mol (dichloroethanol, 1-butanol, 1-pentanol, and trichloroethanol) did not inhibit ATP-activated current. The cutoff in alcohol potency is not explained by the lipid theory of alcohol action and is consistent with an interaction of the alcohols directly with a small hydrophobic pocket on the receptor protein.

#### Cutoff in Alcohol Potentiation of 5-HT<sub>1</sub> Receptor-Ion Channel Function

Previous studies in this Laboratory have shown that ethanol, in a pharmacologic concentration range, can potentiate 5-HT<sub>1</sub> receptor-mediated ion current (Neurosci Lett 1991;122:57-60; Mol Pharmacol 1991;40:263-270). In recent experiments, we investigated the effect of a series of straight chain alcohols from methanol to 1-octanol on the current activated by 1  $\mu$ M 5-HT. In these experiments, methanol, in concentrations from 10 to 500 mM, did not significantly affect the amplitude of 5-HT<sub>1</sub> receptor-mediated ion current ( $P>0.1$ ; n=4), and 100 mM ethanol potentiated 5-HT<sub>1</sub> receptor-mediated ion current in only two of 14 cells tested; the potentiation in those two cells was 5.4% and 21.4%. The straight chain alcohols 1-propanol, 1-butanol, and 1-pentanol, on the other hand, consistently increased the amplitude of 5-HT<sub>1</sub> receptor-mediated ion current in a concentration-dependent manner, with  $EC_{50}$  values of 34.2 mM, 2.7 mM, and 1.4 mM, respectively. Maximal potentiation was  $62\pm 5.1\%$  for 1-propanol (n=6),  $67.5\pm 3.1\%$  for 1-butanol (n=8), and  $85\pm 5.2\%$  for 1-pentanol (n=5). However, despite increased lipid solubility, 1-hexanol did not significantly affect the amplitude of 5-HT<sub>1</sub> receptor-mediated ion current at any concentration between 0.5 and 5 mM (ANOVA;  $P>0.1$ ; n=5), and 1-octanol did not significantly affect the amplitude of 5-HT<sub>1</sub> receptor-mediated ion current at any concentration between 0.01 and 0.5 mM (ANOVA;  $P>0.1$ ; n=4). The highest concentrations of 1-hexanol and 1-octanol tested are equivalent to 7.1 and 6.3 times the membrane alcohol concentration produced by 34.2 mM 1-propanol (the  $EC_{50}$  value for 1-propanol). The cutoff in alcohol potency for potentiation of 5-HT<sub>1</sub> receptor-mediated ion current observed for 1-hexanol and 1-octanol is not explained by the lipid theory of alcohol action and is consistent with an interaction of the alcohols directly with a hydrophobic pocket on the receptor protein. The lack of effect of methanol and ethanol are presumably due to insufficient energy of binding in this hydrophobic pocket to effect the allosteric change in the receptor that results in augmentation of the agonist-activated ion current.



### Cutoff in Alcohol Inhibition of NMDA-Gated Ion Channel Function

Previous studies in this Laboratory have shown that ethanol can inhibit the ion current activated by the glutamate receptor agonist N-methyl-D-aspartate (NMDA) in a concentration-dependent manner over the concentration range 5-50 mM ( $IC_{50}$ =30 mM) (Science 1989;243:1721-1724; Brain Res 1990;507:332-336; J Neurosci 1990;10:1372-1379). In recent experiments, we investigated the effect of a series of straight chain alcohols from methanol to 1-decanol on the current activated by 25  $\mu$ M NMDA. We found that straight chain alcohols from methanol to 1-octanol produced a concentration-dependent inhibition of NMDA-activated current. The  $IC_{50}$  values were: methanol, 351.9 mM; ethanol, 129.8 mM; 1-propanol, 58.6 mM; 1-butanol, 24.4 mM; 1-pentanol, 8.3 mM; 1-hexanol, 4.2 mM; 1-heptanol, 3.4; and 1-octanol, 5.8 mM. However, 1-nonanol and 1-decanol did not inhibit NMDA-activated current, even though these concentrations would result in membrane alcohol concentrations equivalent to those produced by over 3 M and 1.9 M ethanol, respectively. Analysis of these observations indicates that for alcohols with up to five carbon atoms, the potency of the alcohols for inhibiting NMDA-activated current increases exponentially and the increase is significantly correlated with both membrane/buffer partition coefficient ( $r=0.9951$ ;  $P<0.001$ ) and the membrane disordering potency ( $r=0.9969$ ;  $P<0.005$ ) of the alcohols. However, despite increasing membrane/buffer partition coefficient and disordering potency, alcohol potency for inhibiting NMDA-activated current leveled off and then decreased as the number of carbon atoms was increased from six to eight. Moreover, alcohols with nine and 10 carbon atoms did not inhibit NMDA-activated current despite increased lipid solubility and high membrane disordering potency. This cutoff in potency is not explained by the lipid theory of alcohol action because both the membrane/buffer partition coefficient and the lipid disordering potency of the alcohols continue to increase exponentially in the range of the cutoff. On the other hand, the cutoff in potency is consistent with the alcohols inhibiting these neurotransmitter receptors by interacting with a hydrophobic pocket of circumscribed dimensions on the receptor protein. In addition, since 1-nonanol had no effect on NMDA-activated current and the molecular volume of 1-nonanol is about 103.5  $cm^3/mol$ , we estimate that the molecular volume of the putative hydrophobic pocket on NMDA receptors is  $<103.5$   $cm^3/mol$ .

Although the behavioral effects of alcohols are thought to result from alterations in the function of proteins in the central nervous system, the proteins responsible for those effects have not been determined. Previous studies on intoxication, using either loss of righting reflex (J Pharmacol Exp Ther 1981;218:669-675) or ataxia (Neuropharmacology 1978;17:451-461) as the experimental index of intoxication, have shown that as the number of carbon atoms in the alcohol is increased from six to eight, the intoxicating potency of alcohols levels off and then declines, despite increased membrane/buffer partition coefficient and membrane disordering potency. Moreover, the cutoff for alcohol intoxication is similar to the cutoff for alcohol inhibition of NMDA receptors, suggesting that the cutoff for alcohol inhibition of NMDA receptors may contribute to the cutoff for alcohol intoxication, which supports the hypothesis that alcohol inhibition of NMDA receptors may be involved in alcohol intoxication.

### Significance to Biomedical Research and the Program of the Institute:

The cellular mechanisms of alcohol actions in the nervous system have not been established. For almost a century, alcohols have been thought to produce their effects by actions on the membrane lipids of central nervous system neurons. This "lipid theory" of alcohol action attributes alternations in the function of membrane ion channels, receptors, and other membrane proteins to perturbation of membrane lipids. Our observations showing that there are cutoffs in the potency of alcohols for affecting the function of ATP, 5-HT, and NMDA-gated ion channels suggest that alcohols affect the function of these neurotransmitter receptors by interacting directly with a circumscribed hydrophobic pocket on the receptor protein. In addition, the difference in the cutoffs for different receptors suggests that the size of the hydrophobic pocket is different on different types

of receptors.

#### Proposed Course:

The effect of alcohols on other neurotransmitter receptors will be studied to determine whether there are cutoffs in the potency of alcohols for affecting the function of all of these membrane proteins that are alcohol sensitive. In addition, the recognition that alcohols can affect the function of neuronal membrane receptors by interacting with a circumscribed hydrophobic pocket provides a basis for investigating the molecular sites of alcohol action on these membrane proteins, which will also be studied.

#### Publications:

Li C, Aguayo L, Peoples RW, Weight FF. Ethanol inhibits a neuronal ATP-gated ion channel, *Mol Pharmacol* 1993;44:871-75.

Li C, Peoples RW, Li Z, Weight FF.  $Zn^{2+}$  potentiates excitatory action of ATP on mammalian neurons, *Proc Natl Acad Sci USA* 1993;90:8264-67.

Li C, Peoples RW, Weight FF. Alcohol action on a neuronal membrane receptor: Evidence for a direct interaction with the receptor protein, *Proc Natl Acad Sci USA*, in press.

Lovinger DM, Peoples RW. Actions of alcohols and other sedative/hypnotic compounds on cation channels associated with glutamate and 5-HT<sub>3</sub> receptors. In: Alling C, et al., eds. *Alcohol, Cell Membranes and Signal Transduction in Brain*. New York: Plenum Press, 1993;157-67.

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Weight FF, Peoples RW, Wright JM, Li C, Aguayo LG, Lovinger DM, White G. Neurotransmitter-gated ion channels as molecular sites of alcohol action. In: Alling C, et al., eds. *Alcohol, Cell Membranes and Signal Transduction in Brain*. New York: Plenum Press, 1993;107-22.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00480-11 LMCN

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nerve Cell Excitability and Alcohol Actions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: F. Weight

Chief

LMCN, NIAAA

COOPERATING UNITS (if any)

Catholic U, Chile (L. Aguayo); U Pittsburgh Sch. Med. (W. de Groat); Ohio State U (J. Grossie)

LAB/BRANCH

Laboratory of Molecular and Cellular Neurobiology

SECTION

Section on Physiology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Alcohol is classified pharmacologically as a central nervous system depressant. The mechanisms that underlie this alcohol-induced depression of nervous system excitability, however, are poorly understood. This project investigated the intrinsic mechanisms involved in the regulation of nerve cell excitability and the effects of ethanol on those mechanisms. In experiments on the regulation of voltage-gated calcium (Ca++) channels, we found that dopamine (DA) reversibly inhibited both  $\omega$ -conotoxin-sensitive and  $\omega$ -conotoxin-resistant Ca++ currents in superior cervical ganglion neurons. The concentration of DA that induced half-maximal inhibition was 3.0  $\mu$ M. The DA receptor agonists ( $\pm$ )-SKF-38393 (D1 type) and quinpirole (D2 type) appeared unable to induce an inhibition of the Ca++ current. In addition, the DA receptor antagonists SCH-23390 (D1 type) and (-)-sulpiride (D2 type) did not prevent the inhibitory effect of DA. This suggests that the effect of DA on the Ca++ current was not due to activation of DA receptors. The inhibition of the Ca++ current by DA was reduced by application of 1  $\mu$ M phentolamine, a nonselective alpha-adrenergic antagonist, and by prazosin and yohimbine, alpha-1 and alpha-2 adrenergic receptor antagonists, respectively. The beta-adrenergic receptor antagonist propanol did not block the effect of DA. A guanine nucleotide-binding protein appears to be involved in the activation of adrenergic receptors by DA. The addition of GTP $\gamma$ S (0.1 mM) to the intracellular solution produced an effect similar to that of DA. Incubation of the neurons with pertussis toxin reduced the effect of DA by 90%. The results indicate that DA reduces the number of available Ca++ channels in these neurons by activation of alpha-adrenergic receptors, which are associated with a pertussis-sensitive GTP-binding protein. In addition, the effect of ethanol was tested on several types of voltage-gated ion channels in different types of mammalian neurons and it was found to have little or no effect in a pharmacologic concentration range (5 to 100 mM).



Project Description:Investigators:

F. Weight	Chief	LMCN, NIAAA
L. Aguayo	Assistant Professor	Catholic U
W. de Groat	Professor	U of Pittsburgh
J. Grossie	Associate Professor	Ohio State U

Objective:

Although it is well known that the administration of ethanol can affect nervous system excitability, the cellular basis of such actions is poorly understood. The objective of this project was to characterize at the cellular level the intrinsic mechanisms regulating nerve cell excitability and the effects of ethanol on those mechanisms.

Methods Employed:

Excitability mechanisms were investigated by studying voltage-activated membrane ion channels in dissociated and cultured mammalian neurons. The techniques used are briefly described as follows.

Dissociated Neurons

Neurons were dissociated from the nodose, superior cervical and dorsal root ganglia, and from hippocampal and corpus striatal regions of the central nervous system of adult rats using mechanical and enzymatic treatment (Ikeda et al., J Neurophysiol 1986;55:527; Freedman and Weight, Proc Natl Acad Sci USA 1988;85:3618). Electrophysiological recording was obtained from these neurons within a period of ten hours after dissociation.

Cultured Neurons

Hippocampal and cortical neurons were dissociated from 16- to 17-day mouse or rat embryos and grown in culture using the method of Forsythe and Westbrook (J Physiol 1988;396:515). Electrophysiological experiments were performed two to four weeks after plating.

Electrophysiological Recording

In the majority of experiments, membrane ion currents were recorded using the whole-cell patch-clamp method (cf. Ikeda et al., 1986). Receptor agonists and various pharmacologic agents were administered from large-bore micropipettes (>40  $\mu\text{m}$  tip diameter) placed near the cell soma. In experiments recording single channel currents, cell-attached patch-clamp recording was used and pharmacologic agents were in the patch-pipette (cf. Freedman and Weight, 1988).

Data Acquisition

The output of the patch-clamp amplifier was digitized and stored on magnetic media for subsequent analysis using a Compaq 386/20e microcomputer. The microcomputer was also used to generate various patterns of voltage commands.

Major Findings:Regulation of Voltage-Gated Ion Channels

This project investigated the intrinsic mechanisms involved in the regulation of nerve cell excitability and the effects of alcohol on those mechanisms. The whole-cell patch-clamp technique was used to study the regulation of voltage-gated calcium ( $\text{Ca}^{++}$ ) channels. We found that dopamine (DA) reversibly inhibited both omega-conotoxin-sensitive and omega-conotoxin-resistant  $\text{Ca}^{++}$  currents in superior cervical ganglion neurons. The concentration of DA that induced half-maximal inhibition was 3.0  $\mu\text{M}$ . The DA receptor agonists ( $\pm$ )-SKF-38393 (D1 type) and quinpirole (D2 type) appeared unable to induce an inhibition of the  $\text{Ca}^{++}$  current. In addition, the DA receptor antagonists SCH-23390 (D1 type) and



(-)sulpiride (D2 type) did not prevent the inhibitory effect of DA. This suggests that the effect of DA on the  $Ca^{++}$  current was not due to activation of DA receptors. The inhibition of the  $Ca^{++}$  current by DA was reduced by application of 1  $\mu$ M phentolamine, a nonselective alpha-adrenergic antagonist, and by prazosin and yohimbine, alpha-1 and alpha-2 adrenergic receptor antagonists, respectively. The beta-adrenergic receptor antagonist propranol did not block the effect of DA. A guanine nucleotide-binding protein appears to be involved in the activation of adrenergic receptors by DA. The addition of GTPyS (0.1 mM) to the intracellular solution produced an effect similar to that of DA. Incubation of the neurons with pertussis toxin reduced the effect of DA by 90%. The results indicate that DA reduces the number of available  $Ca^{++}$  channels in these neurons by activation of alpha-adrenergic receptors, which are associated with a pertussis-sensitive GTP-binding protein.

#### Ethanol Effect of Voltage-Gated Ion Channels

Whole-cell patch-clamp experiments on adult mammalian neurons acutely dissociated from nodose, superior cervical and dorsal root ganglia revealed a variety of voltage-activated membrane ion currents which include tetrodotoxin (TTX)-sensitive and TTX-resistant sodium currents, a low-threshold transient calcium current (LVA or T-type), a high-threshold sustained calcium current (HVA or L-type), a transient voltage-activated potassium current (A current), a sustained voltage-activated potassium current (delayed rectifier), a sustained calcium-activated potassium current (C current), and a sustained voltage-activated potassium current inhibited by muscarine (M current). The proportion of these currents varied in different neurons and not all currents were found in all neurons. Ethanol, in concentrations from 5 to 100 mM, had little or no effect on these voltage-activated membrane ion currents.

#### Significance to Biomedical Research and the Program of the Institute:

The mechanisms that underlie alcohol-induced depression of nervous system excitability are poorly understood. Characterization of the cellular mechanisms that regulate nerve cell excitability and the actions of ethanol on those mechanisms hold the promise of increasing our understanding of the cellular basis of ethanol's actions in the nervous system.

#### Proposed Course:

The mechanisms regulating nerve cell excitability will be characterized more fully, and the actions of ethanol on those mechanisms will be investigated more extensively. In addition, the actions of other alcohols and CNS depressants such as general anesthetics, opiates, and benzodiazepines will be characterized and compared to ethanol.

#### Publications:

Aguiayo LG, Grossie J. Dopamine inhibits a sustained calcium current through activation of alpha adrenergic receptors and a GTP-binding protein in adult rat sympathetic neurons, *J Pharmacol Exp Ther* 1994;269:503-508.

Aguiayo LG, Pancetti FC. Ethanol modulation of the  $GABA_A$ - and glycine-activated  $Cl^-$  current in cultured mouse neurons, *J Pharmacol Exp Ther*, in press.



**LABORATORY OF NEUROGENETICS**



Annual Report of the  
Laboratory of Neurogenetics  
Division of Intramural Clinical and Biological Research  
National Institute on Alcohol Abuse and Alcoholism  
October 1, 1993 to September 30, 1994  
David Goldman, M.D., Chief

To identify genetic loci determining alcoholism vulnerability, the Laboratory of Neurogenetics is testing for linkage and association between genetic markers and alcoholism and related behavioral phenotypes. It is also engaged in direct gene analyses to detect mutations. Molecular cloning and gene expression techniques are utilized to investigate the neurobiology of genes relevant to ethanol-seeking behavior and in responses to ethanol.

#### Section of Molecular Genetics

The Section of Molecular Genetics studies the structure, control of expression, and variation of genes involved in serotonergic neurotransmission. Serotonin is involved in the regulation of appetite, temperature and sleep, and in impulsive behaviors associated with intolerance to delay. Serotonin has also been implicated in ethanol preference. It is therefore likely that the gene for tryptophan hydroxylase (TPH), the rate-limiting enzyme in serotonin synthesis and genes coding for serotonin receptors, will play a major role in these behaviors. Our studies on TPH have augmented our understanding of its structure, function, expression, and genetic variation. We are extending these studies to identify developmental and tissue-specific regulatory sequences in transgenic mice and in cell lines.

The TPH gene was cloned from mouse mastocytoma cells. The TPH cDNA recognized brainstem TPH mRNA. A TPH polymorphism was identified and used for genetic association studies by D. Nielsen. We reported an association between TPH and suicidality in impulsive Finnish alcoholics. Concentrations of the serotonin metabolite 5-hydroxyindoleacetic acid in CSF have previously been correlated with suicidality and, in this study, were also significantly associated with TPH genotype.

D. Nielsen and associates identified putative regulatory sequences within a 21 kilobase TPH genomic sequence. The mouse TPH promoter was analyzed by mobility shift assay and DNase I footprinting, identifying a novel transcription factor which represses TPH expression in cells, such as P815 mastocytoma, which express large amounts of TPH mRNA. Cognate binding sites of this repressor within the TPH gene were identified and the 27 kb protein was cloned. Deletion analyses of the TPH promoter also detected a second silencer region. This region represses transcription in cells that do not express TPH. Finally, three other regions were located which activate TPH transcription. One of these regions is tissue-specific.

Analysis of the expression of TPH mRNA in several serotonergic cell lines confirmed that both cAMP and dexamethasone regulate TPH expression. A negatively acting glucocorticoid response element (GRE) was identified as well as several conventional GRE sites. These experiments point to a potentially important role for regulation of TPH expression in brain by stress and by neurotransmitters which elevate or reduce cAMP levels in raphe neurons. Alteration of serotonin biosynthesis through such mechanisms could have important behavioral consequences, including effects on ethanol-seeking.

#### Section of Molecular Neurobiology

The Section of Molecular Neurobiology is focusing on the role of DNA methylation in regulation of gene expression in brain. This group is studying DNA methyltransferase, methyl DNA binding protein II (which is expressed in brain), and the demethylase. These enzymes determine the methylation of CpG cytosines and cytosine methylation is generally a mechanism in gene silencing. The



silencing is mediated by methyl DNA binding proteins. Alcohol enhances the expression of many genes and has been reported to inhibit DNA methyltransferase. Therefore, these studies may provide important information on the mechanisms of alcohol's long-term effects on brain function and on fetal alcohol syndrome (FAS) and fetal alcohol effects (FAE).

P. Brooks showed the existence in adult mouse and rat brain of DNA methyltransferase mRNA (by RNase protection assay), DNA methyltransferase protein (by Western blot analysis), and methyltransferase enzyme activity. Highest levels were found in cerebellum and olfactory bulb. The methyltransferase activity is highest in the embryo, where DNA synthesis is most rapid, and also in tissues such as the thymus, which have high rates of cell proliferation and DNA synthesis. However, DNA methyltransferase levels are also quite high in adult brain despite the fact that cell division there has virtually ceased. There is evidence that the enzyme responsible for removing methylcytosines from DNA is DNA polymerase  $\beta$ . Colocalization of highest levels of DNA polymerase  $\beta$  activity in the same brain regions suggests that there may be dynamic turnover of DNA methyl groups in brain. Further studies are needed to understand the role of DNA methyltransferase and methylation/demethylation in adult brain and the effects of inhibiting this enzyme with ethanol both in the adult brain and in the embryo.

#### Section of Molecular Biology

The Section of Molecular Biology focuses on the regulation of several enzymes involved in intermediary metabolism: ethanol-inducible cytochrome P450IIE1 (P450IIE1) and three thiamin-dependent enzymes, pyruvate dehydrogenase,  $\alpha$ -ketoglutarate dehydrogenase, and transketolase.

Recent data demonstrate that elevation of cytochrome P450IIE1 activity positively correlates with higher incidence of liver pathogenesis possibly due to involvement of P450IIE1 in the generation of free radicals and peroxides of long chain fatty acids. During this past year, Drs. Lee and Jeong studied the effect of a synthetic inhibitor of P450IIE1, YH439, which was developed as an experimental anti-cirrhotic agent. Nuclear run-on analysis demonstrated that P450IIE1 was transcriptionally inhibited by this agent whereas other known inhibitors of P450IIE1 competitively inhibited P450IIE1 activity. The molecular mechanism of transcriptional activation of another cytochrome P450, P450IA1 by YH439, was also studied. The effect of YH439 on liver pathogenesis is being studied in a rat model of alcohol-related liver diseases.

The detrimental effect of alcohol during pregnancy is well documented FAS or FAE. Alcohol is considered a teratogen or morphogen since it can cause fetal death or abnormal growth (development) including craniofacial abnormalities. Members of our Section first observed the suppression of various cytochromes P450 and their elevation by alcohol or phenobarbital during pregnancy. The mechanism of pretranslational suppression of hepatic P450IIE1 during pregnancy and its elevation by alcohol drinking was studied. We are exploring the possible roles of cytochromes P450 and their alteration during pregnancy in FAS or FAE.

In collaboration with Drs. Roberts and Shoaf, LCS, NIAAA, the presence of P450IIE1 in brain and other extra-hepatic tissues and its elevation by alcohol drinking were confirmed by immunoblot analyses. Dr. Wang purified P450IIE1 from brain mitochondria and microsomal fractions by immunoaffinity column chromatography and is currently determining the N-terminal sequences of the purified P450IIE1 proteins. To determine the role of brain P450IIE1 in alcoholic brain damage, cDNA clones for the brain P450IIE1 were isolated by Dr. Jeng and their nucleotide sequences are being determined. She plans to over-produce the P450IIE1 proteins from the respective cDNA clones of brain and liver to compare their functional activities.

Dr. Soh successfully purified transketolase from rat liver cytosol and analyzed covalent modification of the purified enzyme. He observed rapid phosphorylation of transketolase by protein kinase C with concurrent inactivation of the enzyme. Other covalent modification like glycosylation was excluded since no changes in the enzyme activity was observed after treatment with endoglycosidase F and glycosidase E. The phospho-amino acid analysis of  $^{32}\text{P}$ -labeled transketolase further verified that only threonine residue is phosphorylated by protein kinase C. Casein kinase II and cAMP-dependent protein kinase, in contrast, very weakly or inefficiently phosphorylated transketolase. The role of phosphorylation of transketolase is being studied in fibroblasts from Wernicke-Korsakoff patients.

Using the cDNA clone we have isolated and polyclonal antibodies, a liver-specific elevation of transketolase was demonstrated during neonatal rat development. In thiamin-deficient rats, the activity of transketolase was significantly reduced while the levels of other thiamin-dependent enzymes such as pyruvate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase were not changed, indicating differential regulation of thiamin-dependent enzymes during thiamin deficiency. Northern mRNA blot analysis further verified that its steady-state mRNA level of transketolase remained comparable to the control animals, suggesting that the reduced transketolase activity was due to its rapid degradation.

Dr. Jeng successfully over-produced catalytically active pyruvate dehydrogenase (PDH) E1 protein in bacteria. This was achieved only after co-expression of E1 $\alpha$  and E1 $\beta$  subunits of the PDH complex in the host bacteria. Using this model, Drs. Kallarakal and Jeng are currently over-producing several PDH mutant proteins that naturally occur in human patients.

In collaboration with Dr. Huh, a former member of the Section, cDNA clones for mitochondrial NADP $^{+}$ -specific and  $\alpha$  subunit of NAD $^{+}$ -specific isocitrate dehydrogenases were identified and characterized. The catalytically active isocitrate dehydrogenases are being over-produced by co-expression of the cDNAs for  $\alpha$  and  $\gamma$  subunits of NAD $^{+}$ -specific isocitrate dehydrogenase.

#### Section of Human Neurogenetics

Because alcoholism is common, complex, and genetically heterogeneous, the Section of Human Neurogenetics has focused its efforts on relatively homogeneous populations and families with more extreme and more narrowly defined phenotypes. Impulsive, alcoholic Finns and their families were studied collaboratively with M. Linnoila and investigators at the University of Helsinki. A collection of more than 600 cell lines and psychiatric evaluations from these families has now been made. Similar collections have been made from more than 500 Pima Indians and more than 200 Cheyenne Indians. These materials have already been useful in studies on the DRD2 dopamine receptor gene, which had been hypothesized to be associated with alcoholism. Using the direct gene analysis approach, four amino acid substitutions at three candidate genes for alcoholism vulnerability have been identified. These genes are 5-HT $_{1A}$  and 5-HT $_{2C}$  serotonin receptors and transketolase.

In ethnically well defined, psychiatrically interviewed groups of alcoholics and controls, there was no association of DRD2 either to alcoholism, to other clinical phenotypes such as early onset, to family history of alcoholism, and to severity, or to CSF homovanillic acid (a crude index of brain dopamine turnover). Linkage disequilibrium between the DRD2 Taq1 marker (10 kb downstream from DRD2) and a second variant in the immediate 3' region of the gene varied between populations but was approximately 35% in two Caucasian populations. Thus, population association with the linked DRD2 marker has limited strength to exclude or include DRD2 as an alcoholism locus. Four-fold interethnic differences in DRD2 marker frequencies were found, which could lead to detection of a spurious DRD2 association. A collaborative study with P. Gejman (NIMH), who used denaturing gradient gel electrophoresis, detected several rare amino acid substitutions but not variants at DRD2, which could account for an authentic association.

The DRD4 dopamine receptor possesses a highly polymorphic 16 amino acid repeating motif coded by exon 3. M. Adamson searched for a functional correlation between DRD4 alleles and alcoholism due to the role of dopamine in reinforcement. In a large population of psychiatrically interviewed Finnish alcoholics and controls, there was no relationship between DRD4 alleles and either alcoholism or concentration of CSF homovanillic acid. M. Adamson also found extensive DRD4 16 amino acid repeat polymorphism in certain other primate species, such as the Orangutan (*Pongo pygmaes*), whereas other species, such as the rhesus macaque (*Macaca mulatta*), were monomorphic. The phylogenetic analysis indicates that the ancestral DRD4 repeat length for the ape lineage is likely to have been five or less. Absence of DRD4 repeat lengths of greater than ten across many primate species is evidence for negative selection against such longer repeat lengths.

A highly informative tetranucleotide repeat polymorphism was detected at the GABA<sub>A</sub>  $\beta 1$  receptor gene and used to map this gene, by genetic linkage, to the centromeric region of chromosome 4. This marker will be particularly useful for linkage studies focusing on GABA<sub>A</sub> receptor function due to the fact that a cluster of GABA<sub>A</sub> receptor genes has been shown (by others) to exist at this location.

The single-strand conformational polymorphism (SSCP) technique was used to detect polymorphisms at TPH (see above), ALDH2 (see below), transketolase (see below), and at eight serotonin receptors: 5-HT<sub>1D</sub>, 5-HT<sub>1D</sub>, 5-HT<sub>1A</sub>, 5-HT<sub>1E</sub>, 5-HT<sub>1F</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, and 5-HT<sub>2</sub>. Multiple polymorphisms were discovered at several of these genes. For example, there were five polymorphisms within the coding sequence of the 5-HT<sub>2A</sub> receptor. Two 5-HT<sub>1A</sub> variants discovered by B. Nakhai are rare amino acid substitutions (22Gly-->Ser and 28Val-->Ile), one conservative and the other nonconservative. A 5-HT<sub>2C</sub> variant found by J. Lappalainen is common (allele frequency=0.13) and nonconservative (23Cys-->Ser).

Polymorphisms were used to genetically map these genes. The 5-HT<sub>1D</sub> gene was localized on 1p35. The 5-HT<sub>1D</sub> and 5-HT<sub>1E</sub> genes were mapped in close proximity to the region of chromosome 6q14-15 and are the first members of this gene family to show colocalization. 5-HT<sub>1F</sub> was mapped on Chr 3. The 5-HT<sub>2C</sub> gene was mapped to its location at Xq21 on the X chromosome.

TPH was mapped, by linkage analysis, to a region of mouse chromosome 7 and, in the human, to chromosome 11p15.5, in the same area which contains tyrosine hydroxylase and which (controversially) has been implicated in affective illness.

The oxytocin receptor gene has been implicated in the biology of affiliative behaviors and anxiety. An oxytocin receptor gene polymorphism was discovered with allele frequencies of 0.77 and 0.23 by S. Michelini. This gene was mapped to chromosome 3p25-26 near the loci for von Hippel-Lindau Disease and renal cell carcinoma.

Studies on genes involved in metabolism have recently been focused on transketolase and aldehyde dehydrogenase (ALDH). The Oriental ALDH2 allele results in enzyme deficiency with alcohol-associated flushing, due to a G->A transition 12 bp from the 3'-end of exon 12. Allele-specific amplification of exon 12 of ALDH2 by A. Novoradovsky showed that the Oriental allele is apparently absent in South American Indians in whom ALDH2 deficiency has been reported. Absence of ALDH2 activity in some South American Indians was confirmed. The search for ALDH2 mutations in South American Indians is therefore being continued to determine the origin of ALDH2 deficiency in South American Indians.

Transketolase has been hypothesized to be defective in individuals at genetic risk for Wernicke-Korsakoff syndrome, which occurs as a result of thiamine deficiency and in some but not all alcoholics. T. Moretti tested the transketolase hypothesis for Korsakoff's disease by SSCP analysis for mutations in 20 Korsakoff's patients. Four SSCP polymorphisms were found. Neither these nor a fifth polymorphism which is an RFLP were associated with Korsakoff's disease in this small sample. A rare 394Thr-->Met substitution was found. This variant is being evaluated for functionality in an *in vitro* expression model.



A population study was completed on the low voltage alpha (LVA) EEG phenotype, a genetic neurophysiologic variant. The LVA trait is relatively common and was found in approximately 5% of controls. The LVA phenotype was more than four times as common in individuals who had alcoholism and was even more abundant in subjects with an anxiety disorder. At least in some families, the LVA trait is transmitted in autosomal dominant fashion. Alcoholism families with LVA are being collected for linkage studies with dispersed genetic markers.

A sample of 159 Cheyenne Indians was evaluated by R. Robin for comorbidity of psychiatric disorders with alcoholism. This sample, consisting of community volunteers and their relatives, included 60 males, of whom 68% were alcoholic, and 99 females, of whom 38% were alcoholic. Although not an epidemiologic survey, lifetime prevalence of alcoholism (49%) is consistent with high reported prevalence of alcoholism among the Cheyenne and the comorbidity rates would be generally reflective of rates in community alcoholics, as opposed to treatment samples. High rates of major psychiatric disorder (56%) and multiple psychiatric disorders (20%) were observed among these community alcoholics, as opposed to nonalcoholics, 18% of whom had a major lifetime psychiatric disorder and 4% of whom had multiple psychiatric disorders. Depression was increased in alcoholic males, indicating a possible difference with female alcoholics, who did not show an increase. All Cheyenne subjects with antisocial personality were alcoholic males and there was a strong relationship between alcoholism and drug abuse. Regarding pattern of drinking, only approximately 10% of the subjects drank heavily without binge episodes, 38% did neither, and the remainder did both. Binge/heavy drinkers were more likely to be divorced or separated, to have few close social or familial relationships, to develop physical symptoms of alcohol abuse and to experience withdrawal symptoms, to have conflicts with the police and to act aggressively, to develop alcoholism at an early age, and to engage in heavy drinking over a longer period of time as compared to subjects who drank heavily and consistently but who did not binge.

For linkage and population genetic analyses, J. Long and M. Urbanek have used the ABI373A sequencer and fluorescence labeling to simultaneously type panels of highly informative short tandem repeat (STR) markers. STR panels consisting of 45 markers have already been analyzed across a variety of populations, including Cheyenne Indians, Pima Indians, Navajo Indians, and Bethesda Caucasians. These analyses revealed that heterozygosity values (and thus informativeness for linkage) were only slightly reduced in the isolated populations.

For linkage analysis, we have collected families from American Indian populations in which alcoholism is highly prevalent. Two hundred Cheyenne Indian and 500 Pima Indian subjects from large families were clinically evaluated and their cell lines immortalized with target family sizes of 600 from each community. The greater environmental and genetic homogeneity of these American Indian communities increases the likelihood of mapping genetic loci or identifying predisposing factors. The families and populations will also be useful for testing the generalizability of other genetic linkages in American Indian communities in which alcoholism accounts for more than 50% of deaths.





Publications  
Laboratory of Neurogenetics  
October 1, 1993 to September 30, 1994

Adamson MD, Kennedy J, Petronis A, Dean M, Virkkunen M, Linnoila M, Goldman D. DRD4 dopamine receptor alleles and CSF HVA and 5-HIAA, Neuropsychiatric Genetics, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00036-08 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Ethanol-Inducible Cytochrome P450 Gene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	B. Song	Section Chief	LNG, NIAAA
Others:	K. Jeong	Visiting Fellow	LMMB, NIAAA
	J. Lee	IRTA Fellow	LNG, NIAAA
	B. Roberts	Visiting Fellow	LCS, NIAAA
	S. Shoaf	Senior Staff Fellow	LCS, NIAAA
	X. Wang	Visiting Fellow	LNG, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Biology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have previously isolated both the cDNA and genomic clones encoding the ethanol-inducible cytochrome P450IIE1 and have demonstrated six distinct types of regulation of its expression. During the past year, we have investigated the effect of an experimental anti-cirrhotic agent YH439 and have demonstrated another mechanism of P450IIE1 regulation. P450IIE1 activity and protein level were effectively suppressed by YH439 in a time- and dose-dependent manner. Nuclear run-on transcription assays confirmed that YH439 inhibited transcription of P450IIE1 gene. In contrast, P450IA1/2 genes were transcriptionally induced by YH439. To elucidate the mechanisms of the transcriptional regulations by YH439, the promoter regions of P4502E1 and P450IA1/2 genes are being actively studied for negative and positive elements, respectively. Gel mobility shift data indicated that YH439 does not mediate its action via the previously characterized XRE (xenobiotic-responsive element), indicating a novel mechanism of induction. We recently published that P450IIE1 was pretranslationally suppressed during pregnancy and that it returned to normal level (within 1 day) upon parturition. We have also investigated the expression of other P450s during pregnancy and their mechanism of gene expression. Preliminary transcription analysis revealed that P450IIE1 was mainly regulated at the posttranscriptional level. Mechanisms of regulating the P450 mRNA turn-over rates are being studied. To verify the presence of P450IIE1 isoenzyme in the brain, P450IIE1 from bovine brain microsomes and mitochondria was purified by affinity column chromatography and the N-terminal amino acid sequences of the purified P450IIE1 proteins are being determined. In collaboration with Dr. Roberts, the levels of P450IIE1 and other P450 isoenzymes in liver, brain and other tissues were studied during alcohol consumption and after alcohol withdrawal period. Under alcohol influence, the levels of P450IIE1 were elevated four- to five-fold in all tissues examined. After withdrawal of alcohol, P450IIE1 level rapidly (within 12 hours) returned to the normal level, supporting our earlier report on the relatively short half-lives of P450IIE1 and its protein stabilization by ethanol and acetone.



Project Description:Investigators:

B. Song	Section Chief	LNG, NIAAA
K. Jeong	Visiting Fellow	LMMB, NIAAA
J. Lee	IRTA Fellow	LNG, NIAAA
B. Roberts	Visiting Fellow	LCS, NIAAA
S. Shoaf	Senior Staff Fellow	LCS, NIAAA
X. Wang	Visiting Fellow	LNG, NIAAA

Objectives:

Ethanol-inducible P450IIE1 has been known to be regulated by several inducers and inhibitors. Unlike other classes of cytochromes P450 which are transcriptionally activated by their respective inducers, the regulatory mechanism of P450IIE1 has been an exception. In general, its activity is regulated via posttranscriptional activation without changes in its mRNA level by various exogenous inducers (ethanol, acetone, isoniazid, pyrazine, imidazole, pyrazole, pyridine) as well as inhibitors (CCl<sub>4</sub>, diallyl sulfide and diallyl sulfone). Because of the pathobiological roles of P450IIE1, a specific inhibitor of P450IIE1 would be highly desired. In this study, the inhibitory mechanism of an experimental anti-cirrhotic compound YH439 on P450IIE1 and other P450s was studied by molecular approaches. The biological roles of P450IIE1 suppression during pregnancy and its activation by alcohol were studied to correlate with the changes in the levels of retinoic acid and its metabolites, which may be involved in the fetal alcohol syndrome (FAS) or fetal alcohol effects (FAE).

Methods Employed:

Animals were treated with YH439 (oral administration) and sacrificed at different times. The microsomal fractions from liver and kidney of control and treated rats were prepared by the differential centrifugation. The washed microsomes were used for measuring N-nitrosodimethylamine demethylase (P450IIE1) and ethoxzoresorufin deethylase activities (P450IA1). The same microsomes were also analyzed by immunoblot analyses using specific antibodies against the respective forms of P450. The total cellular RNA from the same animal groups were prepared for the measurement of the mRNA levels of various P450s by Northern blot analyses using specific DNA probes for P450IIE1 and other P450s. Nuclei from animals were isolated by sucrose pad method and nuclear run-on transcription analyses were performed for P450IIE1, P450IA1/2, and actin cDNA probes to determine transcriptional activity. In order to determine whether an isoform of P450IIE exist in the brain, P450IIE1 in the mitochondria and microsomes were purified using immunoaffinity columns. The immunoaffinity purified proteins from the two subcellular loci were subjected to SDS-polyacrylamide gel electrophoresis, transferred to PVDF-Immobilon membrane and subjected to N-terminal amino acid sequence analyses. Another group of animals were sacrificed at different stages of pregnancy with and without treatment of alcohol and phenobarbital. Hepatic microsomes, total RNA and nuclear fractions were prepared for the enzyme activities, immunoblot, Northern, and nuclear transcription analyses, respectively. Under yellow light, whole blood was taken in the presence of heparin and plasma was then prepared and stored at -80°C. The concentrations of retinoic acid and its structural analogues are being measured by HPLC.

Major Findings:Transcriptional Suppression of P450IIE1 by a Synthetic Inhibitor, YH439

The molecular mechanism of P450IIE1 suppression by a new synthetic inhibitor, YH439, was studied by measuring enzyme activity, and by immunoblot, Northern blot, and nuclear run-on transcriptional analyses. YH439 efficiently inhibited P450IIE1 in a time- and dose-dependent manner. Within two hours after a single administration (oral) of YH439, more than 50% of P450IIE1 activity was inhibited

and the inhibition persisted at 48 hours until P450IIE1 activity returned to the control level. Unlike competitive inhibitors of P450IIE1 such as diallyl sulfide and diallyl sulfone, P450IIE1 inhibition by YH439 was accompanied by concomitant decreases in its protein and mRNA levels. Nuclear transcription analyses confirmed that P450IIE1 was transcriptionally suppressed by YH439, demonstrating that P450IIE1 is regulated by a novel mechanism. To study the mechanism of the transcriptional inhibition, Dr. Wang is preparing several deletion mutants connected a reporter gene (e.g., luciferase) to identify cis-acting element(s) in the 5'-promoter region of the P450IIE1 gene. A manuscript describing our results is being prepared for publication.

#### Transcriptional Induction of P450IA1/2 Genes by YH439

On the other hand, YH439 potentially induced the expression of P450IA1/2. Between two and eight hours after a single administration of YH439, P450IA1/2 levels were induced by more than fifty-fold, comparable to the degree achieved by other potent inducers of P450IA1/2 such as 3-methylcholanthrene and benzo(a)pyrene. The activation of P450IA1/2 by YH439 was the results of concomitant elevation of its protein and mRNA levels. Nuclear run-off transcription analyses revealed that P450IA1/2 genes were transcriptionally induced by YH439. However, the mechanism of P450IA1/2 induction by YH439 is different from that by 3-methylcholanthrene and benzo(a)pyrene. In the latter case, pretreatment with phorbol ester, protein kinase C activator, prevents the transcriptional activation. The binding of inducers to a so-called AH-receptor and translocation of the AH receptor-ligand complex into nucleus are required to initiate its inducing effects via XRE or ARE (anti-oxidant regulatory element) components in the 5'-promoter regions of P450IA1/2 genes. Phorbol ester treatment did not prevent the P450IA1/2 induction by YH439. Gel retardation analyses with nuclear extracts from control and YH439-treated animals did not reveal any shift in the mobility of the respective oligomer of XRE and ARE. These data suggest that YH439 may induce P450IA1/2 genes via a unique, unprecedented mechanism. A manuscript describing our results is being prepared for publication.

#### Pretranslational Regulation of Various Cytochromes P450 During Pregnancy

We recently published that P450IIE1 is pretranslationally suppressed during pregnancy and its activity rapidly returns to normal level upon parturition. We expanded our studies to examine the regulation of other P450s during pregnancy. Our data indicated that other P450s such as P450IA1/2, P450IIB1/2 are also suppressed while P450IV1 level is activated. The changes in these P450-related enzyme activities were paralleled by concomitant changes in their mRNA levels, indicating pretranslational regulation. Preliminary nuclear run-off analyses revealed that these P450s, including P450IIE1, are mainly regulated at the posttranscriptional level. Dr. James Lee is actively studying the mechanism of the posttranscriptional suppression of P450IIE1.

#### Correlation Between Changes in P450IIE1 and P450IIB1/2 and Fetal Alcohol Syndrome

Recent data suggest that ethanol-inducible P450IIE1 and P450IIB1/2 can metabolize arachidonic acid and retinoic acid. Our recent data suggest that P450IIE1 and P450IIB1/2 were inducible by ethanol or other inducers despite their gradual decline during pregnancy, indicating that the P450 induction mechanisms are intact. The induction of P450IIE1 and P450IIB1/2 by alcohol during the pregnancy may be contrary to the natural physiology and lead to marked alteration in the metabolism of endogenous substrates such as long chain fatty acid and retinoic acid, which is absolutely necessary for growth and development. However, too little or excess amount of retinoic acid could be detrimental to normal fetal growth or toxic to the growing embryos, respectively. The abnormal metabolism of these endogenous substrates caused by alcohol drinking during pregnancy may lead to harmful or teratologic effects on fetuses as observed in FAS or FAE. To establish a positive correlation between altered levels of retinoids and FAS (or FAE), we need to measure the levels of these metabolites in plasma and fetal tissues taken at different stages of pregnancy from each animal in the absence or presence of alcohol. The plasma and tissue levels of retinoic acid and its metabolites are being determined by HPLC under the yellow light.

### Regulation of P450IIE1 in Extra-Hepatic Tissues During Alcohol Intake and After Alcohol Withdrawal

In collaboration with Drs. Roberts and Shoaf, LCS, NIAAA, the induction of P450IIE1 by alcohol in extra-hepatic tissues were examined by immunoblot analyses. Immunoblot data using specific antibodies suggest that P450IIE1 is present in the microsomes of kidney, brain, and intestine and that its level is elevated after chronic (three week) alcohol consumption. However, the elevated P450IIE1 returned to the control level at 12 hours after withdrawal of alcohol, indicating a rapid turn-over rate of P450IIE1. Our new findings, observed in all tissues examined, confirmed our earlier result of biphasic half-lives of P450IIE1 (seven and 37 hours) and its protein stabilization by exogenous inducers. Our data disproved a previous report by other investigators of a single half-life of P450IIE1 (25 hours). A manuscript on our data was submitted for publication.

### Purification of Brain P450IIE1 and Characterization

We recently published a paper that brain microsomes contain catalytically active P450IIE1 and that its level is elevated after chronic alcohol consumption. Results from other laboratories suggest that P450IIE1 is also found in the mitochondria, indicating the presence of the P450IIE1 isoenzyme in the brain. Since the microsomes and mitochondria use different peptide leader sequences, the presence of P450IIE1 in the two different subcellular loci suggest that P450IIE1 may be encoded by two similar but different genes. To verify this hypothesis, we are in the process of sequencing the N-terminal amino acids of the purified P450IIE1 proteins from the two organelles. After joining our Laboratory in May, Dr. Wang prepared bovine brain mitochondria and microsomal fractions. She purified P450IIE1-related protein by using antibody-affinity column chromatography. The purified P450IIE1 proteins appeared to reveal one (from microsomes) or two discrete bands (from mitochondria) by Coomassie blue staining of SDS-polyacrylamide gels. The N-terminal amino acid sequence of the immunopurified P450IIE1 bands are being determined by an ABI protein sequencer. In connection, several cDNA clones for brain P450IIE1 were isolated and are being characterized for their structure. The catalytic activities of the over-produced protein from the brain P450IIE1 cDNA clones will be compared to that of the liver enzyme.

### Significance to Biomedical Research and the Program of the Institute:

Chronic alcohol drinking causes numerous changes in cellular functions. In liver, it causes changes in cellular redox states and induces microsomal proteins including ethanol-inducible cytochrome P450IIE1. As we previously reported, it is regulated by many different mechanisms. The multiple types of regulation of P450IIE1 provided a unique example for the P450s, most of which are induced by transcriptional activation, and sets an interesting example for protein regulation in general. Since P450IIE1 is involved in the metabolism of various substrates including carcinogens and clinically used drugs, the induction of P450IIE1 may predispose individuals to certain adverse reactions frequently observed in alcoholic patients.

Recent data strongly suggest that alcohol-induced liver injury may be secondary to the action of P450IIE1 which generates lipid peroxide and oxygen free radicals when it metabolizes arachidonic acid. For these reasons, the elucidation of the molecular mechanism of P450IIE1 regulation and the hepatoprotective effect of a synthetic compound (YH439) are critically important for the mission of the NIAAA. In addition, the alteration of P450-mediated enzyme activities by alcohol during pregnancy may be related to the symptoms observed in FAS or FAE. More careful study will provide better insight into the pathoetiology of FAS and other pathology resulting from alcohol consumption. Furthermore, it will be very interesting to determine the pathophysiological role of P450IIE1 in brain.



Proposed Course:

The regulation of other P450s by alcohol during pregnancy will be studied. Immunoblot, Northern blot, and nuclear transcription analyses will be performed to elucidate the different mechanisms of P450 regulation during pregnancy in the absence and presence of alcohol. The plasma and tissue levels of retinoic acid metabolites will be determined by HPLC to establish the possible role of retinoic acid in fetal alcohol syndrome. Serial deletion of the 5'-promoter region of P450IIE1 or P450IIB1/2 genes will be prepared and connected to a reporter gene. The cis-acting DNA element(s) will be identified after transfection of deletion mutants into suitable cells (HepG-2) in the presence and absence of YH439. The long-term beneficial effect of the P450IIE1 inhibitor, YH439, will be studied in experimental models of alcoholic fatty liver, hepatitis and cirrhosis. The primary structure of brain P450IIE1 will be studied by N-terminal amino acid sequencing and cDNA analysis.

Publications:

Casazza JP, Sohn DH, Park KS, Song BJ. Serum acetone and liver acetone monooxygenase activity in pregnant rats, fetuses, and neonates: Reversible pretranslational reduction of cytochrome P450IIE1 (P450IIE1) during pregnancy, Arch Biochem Biophys 1993;309:111-6.

Menez JF, Machu TK, Song BJ, Browning MD, Deitrich RA. Phosphorylation of cytochrome P450IIE1 by calmodulin dependent protein kinase, protein kinase C and cAMP dependent protein kinase, Alcohol Alcohol 1993;28:445-51.

Song BJ, Soh YJ, Jeng JJ, Goldman D, Lee J. Biochemical properties and multiple regulation of the ethanol-inducible cytochrome P4502E1 subfamily. In: Park SC, ed. Alcohol and Health. Seoul, Korea, 1993;19-29.

Song BJ. Gene structure and multiple regulations of the ethanol-inducible cytochrome P4502E1 (CYP2E1) subfamily. In: Watson RM, ed. Alcohol and Hormones. New Jersey: Humana Press, in press.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00037-08 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Thiamine-Dependent Enzymes Involved in Glucose Metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Song Section Chief LNG, NIAAA

Others: J. Jeng IRTA Fellow LNG, NIAAA  
A. Kallarakal IRTA Fellow LNG, NIAAA  
Y. Soh Visiting Fellow LNG, NIAAA

COOPERATING UNITS (if any)

Kyungpook National University, Korea (T. Huh)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Biology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Because of their importance in energy and intermediary metabolism, we studied the biochemical and molecular characteristics of thiamine-dependent enzymes [the mitochondrial pyruvate dehydrogenase (PDH) complex, the mitochondrial  $\alpha$ -ketoglutarate dehydrogenase ( $\alpha$ -KGDH) complex, and cytosolic transketolase (TK)]. These enzymes were purified to apparent homogeneity from bovine and rat tissues and characterized for their properties and mechanisms of regulation. Tissue-specific structural variations in PDH subunits were confirmed by proteolytic digestion and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A hydrophobic fluorescent probe, bis-ANS, potentially inhibited the activities of PDH and  $\alpha$ -KGDH complexes. Conformational changes in these enzymes were demonstrated upon the addition of allosteric regulators such as ATP or ADP. We demonstrated, for the first time, a posttranslational modification (phosphorylation) of purified TK with a concomitant loss of enzyme activity. In addition, PDH-kinase was purified by DEAE-Sephacel and hydroxyapatite columns followed by Mono Q ion exchange on FPLC. The N-terminal amino acid sequences of the catalytic and regulatory subunits of PDH-kinase were determined. A near full-length cDNA clone for TK was cloned and its nucleotide sequence was also determined. Liver-specific activation of TK was demonstrated. Based on the N-terminal amino acid sequences of the purified proteins, cDNA clones for mitochondrial PDH-kinase, and NADP<sup>+</sup>-specific, and NAD<sup>+</sup>-specific isocitrate dehydrogenases were also identified and these are being characterized. In addition, a catalytically active PDH E1 protein was over-produced in *E. coli*. Site-directed mutagenesis of the E1 subunit simulating naturally occurring mutations is being performed to elucidate the molecular mechanism of how structural variations affect the catalytic activity or the stability of the PDH E1 subunit.

Project Description:Investigators:

B. Song	Section Chief	LNG, NIAAA
T. Huh		Kyungpook Natl U, Korea
J. Jeng	IRTA Fellow	LNG, NIAAA
A. Kallarakal	IRTA Fellow	LNG, NIAAA
Y. Soh	Visiting Fellow	LNG, NIAAA

Objectives:

Heavy and long-term alcohol consumption often lead to reduced function and damage of the brain and other organs. These unfortunate changes are partly due to deficiencies in nutrients including many essential vitamins. Changes in energy metabolism have also been suggested to occur during chronic alcohol consumption and in fetal alcohol syndrome. Because of the importance of thiamine-dependent enzymes in energy metabolism especially in brain, the biochemical and molecular mechanisms of the regulation of three thiamine-dependent enzymes (TK, PDH, and  $\alpha$ -KGDH), as well as their abnormal gene expression after ethanol consumption, were studied using specific antibodies and cDNA probes.

Methods Employed:

The mitochondrial PDH complex,  $\alpha$ -KGDH complex, and cytosolic TK were purified from bovine or rat tissues by conventional column chromatographies and to apparent homogeneity. Phosphorylation of TK was carried out in a buffer (500  $\mu$ l) containing 50 mM PIPES, pH 6.5 with 10  $\mu$ g/ml diacylglyceride, 10  $\mu$ g/ml phosphatidylserine, 10 mM  $Mg^{2+}$ , 5 mM  $Ca^{2+}$ , and 1  $\mu$ l protein kinase C (CalBiochem, San Diego, CA) at 37°C for two hours. A small aliquot (50  $\mu$ l) was removed at different times from the reaction tube and heated for five minutes in boiling water bath and in the presence of SDS-sample buffer. The sample was then analyzed by SDS-PAGE and autoradiographed at -80°C.

Presence of phospho-amino acid was determined as follows. Complete hydrolysis of  $^{32}P$ -labeled TK was performed in 6 N HCl for four hours at 110°C. The resulting phospho-amino acid was analyzed by thin layer chromatography on a cellulose acetate sheet. The relative mobility of the phospho-amino acid was compared to those of authentic phospho-amino acid standards: Ser, Thr, and Tyr.

Each subunit of the PDH complex from brain and kidney was further isolated by gel filtration column chromatography. Tissue-specific structural variations were confirmed by determining the mobility of the proteolytic fragments of the various subunits by SDS-PAGE and Coomassie blue staining. A fluorescent probe, bis-ANS was used to study the biochemical properties of the purified  $\alpha$ -KGDH and PDH complexes. The purified proteins were also subjected to N-terminal and internal amino acid sequencing analyses.

Based upon amino acid sequence data from the purified proteins, several oligodeoxynucleotides were synthesized using an oligonucleotide synthesizer (ABI). These synthetic oligomers were used for DNA amplification by polymerase chain reaction following reverse-transcription (RT-PCR). The nucleotide sequence of the PCR-amplified DNA fragments were determined and used to screen cDNA libraries of various tissues from several species including human.

To assess effects of thiamine deficiency on the thiamine-dependent enzymes, rats were placed on thiamine-deficient diet for three weeks prior to sacrifice. Livers, kidneys and brains were quickly excised and either frozen in liquid

nitrogen or used immediately for the preparation of mitochondria or cytosolic fractions by differential centrifugation. Catalytic activities, the levels of immunoreactive proteins, and the mRNA levels of the thiamine-dependent enzymes were determined.

For site-directed mutagenesis studies, a co-expression plasmid containing the coding regions of PDH E1 $\alpha$  and E1 $\beta$  subunits was constructed with PCR-amplified DNA fragments and ligated into an expression vector pT7-7 at FspI/PvuII sites. *E. coli* strain BL21(DE3)/plyss (Novagen, Madison, WI) was used as the host for the over-production. The *E. coli* strain carrying the co-expression plasmid was grown in LB medium at 37°C in the presence of ampicillin (50  $\mu$ g/ml) and chloramphenicol (10  $\mu$ g/ml) until OD<sub>600</sub> reached 0.4. IPTG (0.4 mM) was added to induce the synthesis of T7 polymerase and PDH E1 component. Rifampicin (0.2 mg/ml) was added one hour after induction with IPTG to reduce the synthesis of host proteins. The cells were harvested by centrifugation at 2,500 x g, suspended in 1 x PBS with 0.1% Triton X-100, and disrupted by freeze-thawing and sonication. The cell extracts were centrifuged in a microfuge for 10 minutes and the soluble fraction was used for PDH activity measurement using [<sup>14</sup>C]pyruvate as the substrate.

### Major Findings:

#### Posttranslational Modification of Transketolase (TK)

Singleton et al. (McCool et al., J Biol Chem 1993;268:1397-1404) recently reported the characteristics of a cDNA clone for human liver TK. They observed no sequence variation of TK between normal and Wernicke-Korsakoff (WK) patients, indicating a differential regulation or posttranslational modification of TK protein in the WK patients. These findings have been confirmed in a larger sample of WK patients by investigators in this Laboratory (T. Moretti, D. Goldman). For this reason, we studied the posttranscriptional regulation of TK. Hepatic TK was purified more than 120-fold from the rat liver cytosolic fraction by successive column chromatographies: DEAE-Sephacel, hydroxyapatite, and Mono Q column on FPLC. The purified enzyme was rapidly phosphorylated by exogenously added protein kinase C. The only phosphorylated amino acid of TK was determined to be threonine. The phosphorylated enzyme had a lower activity (approximately 50%) compared to nonphosphorylated enzyme. Similar loss of TK enzyme activity (40%) was also observed *in vivo* animals between one to four hours after treatment with phorbol ester, protein kinase C activator. Our data demonstrate that TK can be phosphorylated *in vivo* by protein kinase C or other protein kinases and that this modification may correspond to the TK variants observed in the patients. A manuscript describing our results is being prepared for publication.

#### Cloning of cDNA Encoding Rat Transketolase (TK)

Using polyclonal antibodies, a near-full length cDNA clone for rat liver transketolase was identified. Complete DNA sequencing of this cDNA clone (DNA insert 2.0 kb long) revealed that the rat TK is 93% similar in amino acid sequence to the human TK reported recently. However, rat TK appears to have an extra 31 amino acid residues at the N-terminus. In order to confirm this finding and to perform biochemical characterization, cytosolic transketolase from rat liver was also purified to near homogeneity as described above. The first six amino acid residues of the purified rat protein determined by N-terminal amino acid sequencing were 100% identical to the cDNA-deduced TK sequence. In addition, a liver-specific pretranslational activation of TK was observed during neonatal rat development. A manuscript is about to be submitted for publication.

#### Over-Production of Catalytically Active PDH Gene

The mitochondrial PDH is a key enzyme connecting glycolysis and the tricarboxylic acid cycle by catalyzing the production of acetyl CoA and CO<sub>2</sub> from pyruvate. It is therefore very important in mitochondrial energy (ATP) production and intermediary metabolism such as biosynthesis of neurotransmitter, acetylcholine, and fatty acid. Deficiency in PDH activity is the leading cause of congenital lactic acidosis resulting in premature death or severe growth or mental



retardation. It is now known that there are many types of structural mutation in the E1 subunit. However, the mechanism of how these mutations affect PDH activity is not known. After cloning the genes for the subunits of the PDH complex, we subsequently studied the biochemical properties of normal and mutated PDH proteins after over-production in *E. coli* using a co-expression plasmid which contains the coding region of both the E1 $\alpha$  and E1 $\beta$  subunits of PDH complex. For the first time, a functionally active PDH E1 protein was produced when this co-expression plasmid was introduced into *E. coli*. However, the over-production of E1 $\alpha$  alone resulted in a catalytically inactive protein, suggesting an important role of the E1 $\beta$  subunit in constituting enzyme activity. The PDH E1 protein produced in bacteria appeared to comprise about 2-3% of the soluble bacterial protein. The E1 $\alpha$  and E1 $\beta$  subunit were co-eluted from a DEAE-Sephrose column, indicating a tight association between them. The over-produced PDH E1 protein could be phosphorylated by PDH-kinase, mimicking *in vivo* PDH regulation. Our data thus indicate that the over-production system will be a useful tool for studying the biochemical properties of human E1 subunit, in which many naturally occurring mutations are found. A manuscript has been submitted for publication.

After joining the Laboratory in March, Dr. Abraham Kallarakal has successfully purified the PDH complex from bovine brain and kidney. Comparison of the proteolytic fragments of the PDH subunits from brain and kidney suggests that PDH E3 subunit and protein X are structurally different in brain versus in kidney. Biochemical analyses of the purified proteins from kidney and brain are currently being conducted to examine the possibility of tissue-specific structural variations of the PDH subunits. In addition, cDNA cloning of these variant proteins in brain cDNA library is being attempted in order to complement the protein structural analyses.

#### Cloning of cDNAs for Mitochondrial NADP<sup>+</sup>- and NAD<sup>+</sup>-Specific Isocitrate Dehydrogenases (ICDH)

Along with  $\alpha$ -KGDH, ICDH is a key enzyme in the mitochondrial tricarboxylic acid cycle. In collaboration with Dr. T.L. Huh, Korea, we recently published a paper describing the characteristics of a cDNA clone for the bovine heart mitochondrial NADP<sup>+</sup>-specific ICDH. We also identified cDNA clones for the human enzyme, which is 97% identical to the bovine enzyme. During the last year, for the first time, we were able to isolate cDNA clones for the mitochondrial NAD<sup>+</sup>-specific ICDH $\alpha$  subunit using degenerative oligonucleotides synthesized from the previously known peptide sequence data. The structural comparison of various ICDH isoenzymes indicates that the mitochondrial NAD<sup>+</sup>-specific ICDH $\alpha$  subunit is more related to yeast IDH2, the catalytic subunit, than yeast IDH1, the regulatory subunit. It was also concluded that this protein is derived from a single gene and mRNA transcript (2.8 kb). Although we were able to over-produce the protein from this cDNA clone in *E. coli*, it was not catalytically active. The inactivity of the over-produced protein could be due to its abnormal assembly with other subunits. Alternatively, another subunit of IDH $\beta$  or IDH $\gamma$  subunit may be needed to constitute IDH activity. This is the case for the mitochondrial PDH complex where the regulatory E1 $\beta$  subunit is required for the catalytic E1 $\alpha$  subunit (as discussed above). In addition, several cDNA clones for IDH $\beta$  and IDH $\gamma$  subunits were identified by RT-PCR (reverse transcription and polymerase chain reaction). The primary structures of these clones are being determined. Production of a catalytically active IDH protein by using a co-expression plasmid containing the coding regions of IDH $\alpha$  and IDH $\beta$  subunits are also being pursued. A manuscript is about to be submitted for publication. A second manuscript is in preparation describing the cDNA cloning for the IDH subunit.

#### Inhibition of Mitochondrial $\alpha$ -KGDH Complex by Bis-ANS

The regulation of  $\alpha$ -KGDH complex was studied because of its rate limiting role in the mitochondrial TCA cycle. The purified  $\alpha$ -KGDH complex was inhibited ( $K_i$  9  $\mu$ M) by a hydrophobic fluorescent probe, bis-ANS. The enzyme inhibition appeared to take place at E1 and E2 subunits since it did not inhibit E3 activity. The fluorescent spectra of bis-ANS and enzyme complex was further increased by the addition of allosteric regulators, indicating conformational

changes in the enzyme complex. Our study further demonstrates that bis-ANS can be a valuable tool to study the interaction of the multienzyme complex and its allosteric regulators. A manuscript describing our results is in press.

#### Differential Regulation of TK, PDH and $\alpha$ -KGDH Complexes During Thiamine-Deficient States

In thiamine-deficient rats, the catalytic activities of TK, PDH and  $\alpha$ -KGDH complexes are decreased. The amounts of immunoreactive TK markedly decreased while those of immunoreactive PDH and  $\alpha$ -KGDH proteins were unaltered, indicating a differential regulation among the three thiamine-dependent enzymes. The turnover rate of hepatic TK is being determined in control and thiamine-deficient rats.

#### Cloning of cDNA Clones for PDH-Specific Kinase

The N-terminal amino acid sequences of the catalytic subunit (PDH-K<sub>1</sub>, M<sub>r</sub> of 48,000) and the regulatory subunit (PDH-K<sub>2</sub>, M<sub>r</sub> of 45,000) of PDH-kinase were determined. Internal amino acid sequences for both subunits were also determined. Based on the N-terminal and internal sequences, several oligodeoxynucleotides were synthesized and used to isolate cDNA clones for both subunits by RT-PCR (reverse transcription polymerase chain reaction). We have amplified several DNA fragments (DNA sizes 0.5-1.0 kb long) for both subunits. The primary structures of the amplified DNA fragments are being determined to verify the correct clones for PDH-kinase subunits.

#### Significance to Biomedical Research and the Program of the Institute:

Chronic alcohol consumption often leads to malnutrition and loss of body weight apparently due to abnormal metabolism of glucose which serves as a major energy source for numerous cellular functions. It was suggested that these changes are due to deficiency in one soluble vitamin, vitamin B<sub>1</sub> (thiamine), which serves as a cofactor for key enzymes involved in glucose metabolism. It was also reported that the activities of TK and  $\alpha$ -KGDH appear to be reduced after prolonged starvation and in Wernicke-Korsakoff patients. Despite numerous reports on the biochemical properties of these enzymes in normal and pathophysiological conditions, the detailed mechanisms of the altered activities of these enzymes are still controversial. Because of the importance of these enzymes in energy metabolism not only in human alcoholism but in the general field of cellular biochemistry, we are studying the molecular regulation of the three major thiamine-dependent enzymes: cytosolic TK, mitochondrial PDH, and  $\alpha$ -KGDH. Our study includes various approaches of biochemical, immunological, and molecular biological techniques. The understanding of these proteins in normal conditions and under the influence of alcohol may also lead to a better understanding of the mechanisms of fetal alcohol syndrome and to better strategies to prevent alcohol-associated tissue damage.

#### Proposed Course:

(1) Using the co-expression plasmid, catalytically active PDH E1 protein was successfully over-produced in bacteria. Site-directed mutagenesis simulating natural mutations will be performed. Using this co-expression system, we will produce a battery of mutant proteins that are naturally occurring in human patients. Our study will elucidate the biochemical mechanism of how these mutations affect PDH activity or stability. Once the mechanisms are revealed, it may be possible to treat patients suffering from lactic acidosis via various gene transfer/therapy techniques.

(2) The rat PDH E1 $\alpha$  isoform will also be over-produced from the cDNA clone. The over-produced rat PDH protein will be subjected to phosphorylation by PDH-kinase isolated from bovine kidney to demonstrate a testis-specific differential regulation of the enzyme. The kidney PDH complex will be used as a positive control. The study will provide a direct evidence that testis isoenzyme may be differentially regulated from that of the kidney enzyme.



The immunologically distinct PDH E1 $\alpha$  present in rat testes and brain will be purified by affinity chromatography using purified polyclonal antibodies against the PDH E1 $\beta$  subunit. The regulatory mechanism of gene expression for the PDH E1 $\alpha$  isoform will be studied using anti-peptide antibodies and specific cDNA probes for the variant. The kinetic properties of the purified enzyme and over-expressed protein derived from cDNA will be compared with those found in other tissues and species. The differential regulation of PDH E1 $\alpha$  isoform will be studied under the conditions in which the regular PDH E1 $\alpha$  is inactivated via phosphorylation by the specific PDH-kinase. These conditions include starvation or treatment of animals with neurotoxic agents as reported earlier.

(3) Isolation of the correct cDNA clones for PDH-kinase and PDH-phosphatase subunits will be continued. The deduced protein sequences will be compared with those in the protein data bank to identify any similarity to other classes of protein kinases or protein phosphatases in order to obtain structural information for the study of reaction mechanisms. The anti-peptide polyclonal antibodies will be also used for the study of the induction mechanisms of these proteins during changes in physiological conditions.

(4) The molecular regulation of purified transketolase will be studied to identify the nature of different isozymes or covalent modifications leading to different isoelectric points.

(5) Using specific antibodies and cloned cDNAs for transketolase and  $\alpha$ -KGDH complex, the molecular mechanisms for the abnormal expression of these enzymes in thiamine-deficient animals will be studied.

(6) The genomic organization of the cDNA clones described above will be studied to characterize the promoter regions necessary for transcriptional regulation during normal development and their changes under pathophysiological conditions. The possibility of genetic polymorphism and differences in abnormal tissues will also be pursued by analyzing restriction fragment length polymorphism for genomic DNAs or specific ribonuclease digestion method in fibroblasts or lymphocytes obtained from human subjects, including alcoholics and Wernicke-Korsakoff patients.

(7) We will collaborate with Dr. P.J. Brooks (Section of Neurobiology, LNG) to study the distribution and expression of these thiamine-dependent enzymes in brain. These studies will utilize *in situ* hybridization techniques and antisense oligonucleotides or RNA techniques to modify the expression of these genes.

#### Publications:

Hong SY, Bak CI, Ryu JH, Song BJ, Huh JW. Inhibition of bovine  $\alpha$ -ketoglutarate dehydrogenase complex by 1,1'-bi(4-aniline)naphthalene-5,5'-disulfonic acid, J Biochem, in press.

Huh TL, Huh JW, Ryu JH, Casazza JP, Veech RL, Song BJ. Characterization of immunologically distinct pyruvate dehydrogenase E1 $\alpha$  subunit in rat testis, Molecules and Cells, in press.

Jeng J, Huh TL, Song BJ. Production of an enzymatically active E1 component of human pyruvate dehydrogenase complex in *E. coli*: Supporting role of E1 $\beta$  subunit in E1 activity, Biochem Biophys Res Commun, in press.

Kim YO, Oh IU, Park S, Jeng J, Song BJ, Huh TL. Isolation of a cDNA clone for NAD $^{+}$ -specific isocitrate dehydrogenase  $\alpha$  subunit and structural comparison with its isoenzymes from different species, Biochemistry, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 00086-01 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Studies on 5-HT1A Receptor Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Nakhai Visiting Fellow LNG, NIAAA

Others: L. Akhtar Chemist LNG, NIAAA

D. Goldman Chief LNG, NIAAA

G. Jenkins Biologist LNG, NIAAA

D. Nielsen Senior Staff Fellow LNG, NIAAA

COOPERATING UNITS (if any)

Program Resources Inc., Frederick, MD (M. Dean); Cedar Sinai Medical Center, Los Angeles, CA (C. Readhead)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Genetics

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.55

PROFESSIONAL:

0.35

OTHER:

1.20

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The serotonin 1A (5-hydroxytryptamine) receptor gene codes for the 5-HT1A receptor. It is expressed both presynaptically in serotonergic cell bodies and in dendrites of the dorsal raphe nucleus and postsynaptically in hippocampus. Serotonin plays a key role in many physiological and behavioral functions, including intolerance to delay, aggressivity, impulsivity, appetite, and control of temperature and sleep. Serotonergic activity is regulated, in part, by binding of serotonin to the 5-HT1A receptor. To define the mechanisms controlling 5-HT1A receptor gene expression, we isolated several overlapping genomic clones from a human genomic library and are constructing gene fusions.

The regulation of 5-HT1A receptor expression is being studied in various tissue culture cell lines. The effects of various drugs on 5-HT1A receptor mRNA content in these cells are being investigated to elucidate factors controlling its expression. Various deletions of the 5-HT1A receptor gene promoter have been fused to the luciferase reporter gene. These are used for transfection into several tissue culture cell lines to identify sequences controlling 5-HT1A receptor gene expression. Regions necessary for tissue-specific expression and regulation by the various drugs are to be identified through the use of various deletion constructs.

Allelic variants of the 5-HT1A gene have been identified by SSCP analysis in both humans and monkeys. Two polymorphisms change the protein sequence of the 5-HT1A receptor and a third is silent. We are studying the relationship of these polymorphisms to impulsive behavior in alcoholic Finn violent offenders and in several other human populations.

Project Description:Investigators:

B. Nakhai	Visiting Fellow	LNG, NIAAA
L. Akhtar	Chemist	LNG, NIAAA
M. Dean	Staff Scientist	PRI, Frederick, MD
D. Goldman	Chief	LNG, NIAAA
C. Jenkins	Biologist	LNG, NIAAA
D. Nielsen	Senior Staff Fellow	LNG, NIAAA
C. Readhead	Director of Transgenics Core	CSMC, Los Angeles, CA

Objectives:

The objective of the Section of Molecular Genetics is to understand the genetic determinants of serotonin function. To accomplish this, we are studying the regulation of serotonin 5-HT<sub>1A</sub> receptor gene expression.

The 5-HT<sub>1A</sub> receptor belongs to the family of G-protein coupled receptors. Expression of the 5-HT<sub>1A</sub> gene in the brain is highest in raphe nucleus, septum, hippocampus, entorhinal cortex, and interpeduncular nucleus. The 5-HT<sub>1A</sub> receptor functions as a presynaptic autoreceptor on serotonergic neurons. The action of the 5-HT<sub>1A</sub> receptor is mediated through several interrelated mechanisms. Cystolic Ca<sup>2+</sup> levels are elevated, neuronal firing is decreased, sodium-dependent phosphate transport is activated, and adenylyl cyclase is inhibited reducing intracellular cAMP levels. Furthermore, binding of serotonin or the serotonin agonist ALK-3 to the 5-HT<sub>1A</sub> receptor inhibits serotonin release.

The 5-HT<sub>1A</sub> receptor has been implicated in alcohol preference and impulsive behavior. In alcohol-preferring rats, density of 5-HT<sub>1A</sub> receptors in frontal cerebral cortex and hippocampus was significantly higher (39% and 131%, respectively) as compared to alcohol-nonpreferring rats. This was accompanied by a concomitant 41% decrease in the KD of the 5-HT<sub>1A</sub> receptor in alcohol-preferring rats. Furthermore, specific 5-HT<sub>1A</sub> agonists, such as 8-OH-DPAT, buspirone and ipsapirone, block isolation-induced aggression in mice, and resident-intruder and maternal aggression in rats.

Low serotonin turnover has been strongly implicated in impulsive behaviors and with alcohol preference. The regulation of this gene's expression and its roles in the control of behaviors in humans and monkeys are therefore being investigated.

Methods Employed:

Genetic cloning and gene constructions were performed by standard procedures. Sequences from human 5-HT<sub>1A</sub> receptor, human metallothionin, firefly luciferase, HSV thymidine kinase, Tn5 neomycin-kanamycin resistance, as well as SV40, RSV, and MMTV promoters and regulatory elements, and pUC sequences, have been combined to form the appropriate vectors for the studies outlined below. Methods used include restriction endonuclease cutting, filling with Klenow enzyme, fragment isolation, ligation, site-specific mutagenesis, and dideoxy sequencing.

5-HT<sub>1A</sub> receptor expressing and nonexpressing cell lines were grown in tissue culture. Cell lines used include Jurkat E-6 cells, a human T cell line, OK (opussum kidney) cell line, COLO320 cell line, Cos-7 cells, TT cells, a human medullary thyroid carcinoma cell line, NIH3T3 cells, a mouse fibroblast line, and P815 cells, a mouse mastocytoma cell line. Some of these cell lines will be transfected with the appropriate DNA plasmid constructions by the calcium phosphate method. Transiently transfected cells as well as stable, G418 resistant cell lines will be analyzed. Gene function will be assayed through the



use of the highly sensitive reporter gene luciferase as well as by mRNA Northern analysis.

For transfection and expression studies, mRNA was extracted from cell lines and tissues and at times was enriched for poly(A)<sup>+</sup> mRNA. The RNA was electrophoresed and transferred to nylon membranes.

Single-strand conformation polymorphism (SSCP) were performed on DNA samples of human and primates. The complete coding region of 5-HT<sub>1A</sub> gene was amplified with primers by polymerase chain reaction, digested with several restriction enzymes, denatured and run on nondenaturing polyacrylamide gels. Bands were analyzed by autoradiography or phosphorimaging and DNA was sequenced to characterize variants.

#### Major Findings:

The partial genomic 5-HT<sub>1A</sub> clone, G21, kindly provided by Dr. M. Caron, was used as a hybridization probe to screen a recombinant  $\lambda$  DASH human genomic library. Several overlapping clones spanning 4 kb of the human gene were isolated and characterized by restriction enzyme mapping. The human 5-HT<sub>1A</sub> coding region is ~1.3 kb in length. The overlapping clones were subcloned in pGEM3Z vector series and sequenced to determine the 5' untranslated and promoter regions. One clone, pHTL1, contained the complete coding sequence and approximately 2 kb of the upstream sequences. BamHI digestion of this clone released a ~4.2 kb fragment containing the putative promoter region. This upstream region was fused upstream of the firefly luciferase gene to form pHTL1, pHTL2, and pHTL3, respectively.

To identify the sequences of the 5-HT<sub>1A</sub> promoter region specific for transcriptional regulation, a series of plasmid constructions have been designed. Deletions of the 5-HT<sub>1A</sub> upstream sequence were fused to the luciferase gene by site directed mutagenesis. Sequence analysis of the 5' upstream regulatory regions revealed the presence of many putative regulatory regions. Regulatory sites are present for Ap-2, Sp1, NF-1, and octamer binding sites as well as general helix-turn-helix protein binding sites. To analyze this plethora of presumptive regulatory sites, we have fused the 5' upstream region of the human 5-HT<sub>1A</sub> genomic clone to the gene coding for firefly luciferase. When transfected into tissue culture cells, a fusion mRNA will be produced of the 5'-untranslated region of 5-HT<sub>1A</sub> followed by luciferase 5 untranslated region. Translation will begin with the luciferase AUG. This fusion allows gene expression to be easily assayed with extreme sensitivity. This DNA construct has been introduced into various tissue culture cells by the calcium phosphate technique along with a control plasmid, CMV- $\beta$ gal, to provide an unregulated, internal transfection efficiency control. The DNAs have been transfected into the 5-HT<sub>1A</sub> synthesizing cell lines, Jurkat E-6 cells, a human T cell. Mouse 3T3 fibroblast cells have been transfected as a nonserotonergic control cell line. Compounds including 5-HT<sub>2A</sub>, dibuteryl cAMP, dexamethasone, and forskolin will be assayed to measure their effect on transcription. To further analyze sites which may control expression, deletions of the 5-HT<sub>1A</sub> promoter are being constructed by taking advantage of convenient restriction endonuclease sites and by site-directed mutagenesis. This will allow the mapping of regulatory sequences at which drugs act as well as sites determining tissue-specific expression.

To find polymorphisms in the 5-HT<sub>1A</sub> gene in humans and in monkeys, we amplified genomic DNA using the SSCP technique. Two rare polymorphisms have been identified which give rise to amino acid substitutions in the human 5-HT<sub>1A</sub> gene. Each has two allelic forms that have been typed by SSCP analysis and sequencing. To assess the functionality of the substitutions, the 5-HT<sub>1A</sub> gene was also subcloned in the eukaryotic expression vector pcDNA3. In pcDNA3 the expression of the 5-HT<sub>1A</sub> coding region is driven by the strong CMV promoter. pcDNA-5-HT<sub>1A</sub> was used to introduce the two identified point mutations. The N-terminal extracellular region will also be deleted to test the role of this domain on

function. These plasmids will be transfected into the Cos-7 cells. Expression of these mutated 5-HT<sub>1A</sub> receptors will allow us to study the effect of the polymorphisms on function. Each amino acid substitution may be an important tool in the study of serotonergically-influenced traits in humans. A third polymorphism found in the coding region of the human 5-HT<sub>1A</sub> gene did not give rise to an amino acid substitution. Allele frequency in Caucasians and American Indians for 5-HT<sub>1A</sub>-1231T was 0.992.

An SSCP polymorphism has also been identified in the 5-HT<sub>1A</sub> coding region of primates (vervet monkeys) gene. This polymorphic region is now being sequenced.

To identify an *in vitro* system to study the regulation of the 5-HT<sub>1A</sub> receptor gene, we assayed various cell lines for 5-HT<sub>1A</sub> content. RNA was isolated from P815, COLO 320DM, TT, Jurkat E-6, and OK cells. To quantitate the expression of the 5-HT<sub>1A</sub> receptor gene in these cell lines, northern hybridization analyses were performed. 5-HT<sub>1A</sub> mRNA was observed in Jurkat E-6 cell line.

#### Proposed Course:

To understand the effects of environmental and physiological factors such as stress on serotonin function and impulsive behavior, we are investigating the regulatory control of 5-HT<sub>1A</sub>. Using transfection of tissue culture cells, we are delineating the factors controlling 5-HT<sub>1A</sub> gene expression. The studies should reveal sequences in the 5-HT<sub>1A</sub> promoter involved in 5-HT<sub>1A</sub> receptor gene regulation as well as those determining tissue-specificity. Once these controlling sites are identified, we will be able to characterize and identify factors mitigating these actions. The 5-HT<sub>1A</sub> polymorphisms we have identified in both humans and monkeys are being characterized further. When the 5-HT<sub>1A</sub> gene is linked to their cognate behaviors, the 5-HT<sub>1A</sub> genes will be isolated. These polymorphic 5-HT<sub>1A</sub> genes will be analyzed to determine how they vary in function and how this controls behavior. Behavior can then be related directly to 5-HT<sub>1A</sub> gene and serotonergic activity.

#### Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00008-02 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on Serotonergic Gene Function and Behavior in Transgenic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. Nielsen	Senior Staff Fellow	LNG, NIAAA
Others:	P. Brooks	Senior Staff Fellow	LNG, NIAAA
	D. Goldman	Chief	LNG, NIAAA
	G. Jenkins	Biologist	LNG, NIAAA
	B. Nakhai	Visiting Fellow	LNG, NIAAA
	K. Schuebel	IRTA Fellow	LNG, NIAAA

COOPERATING UNITS (if any)

Cedars Sinai Medical Center, Los Angeles, CA (C. Readhead)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Genetics

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda MD 20892

TOTAL STAFF YEARS:

.30

PROFESSIONAL:

.15

OTHER:

.15

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Since alcoholism is associated with decreased serotonin turnover, we have focused on genetic determinants of serotonergic behaviors to identify factors contributing to a predisposition to alcoholism. In raphe neurons, serotonin biosynthesis is governed by the activity of the tryptophan hydroxylase (TPH) enzyme, which is rate-limiting. The cDNA and gene coding for murine TPH were previously cloned. These sequences have been combined with sequences from luciferase, HSV thymidine kinase, and mouse metallothionein. These are being introduced in mouse embryos to create transgenic mice to study gene sequences controlling tissue-specific and developmental expression, and to characterize effects of high and low TPH gene activity, and ablation of TPH-expressing cells on behavior.

Project Description:Investigators:

D. Nielsen	Senior Staff Fellow	LNG, NIAAA
P. Brooks	Senior Staff Fellow	LNG, NIAAA
D. Goldman	Chief	LNG, NIAAA
G. Jenkins	Biologist	LNG, NIAAA
B. Nakhai	Visiting Fellow	LNG, NIAAA
C. Readhead	Director of Transgenics Core	CSMC, Los Angeles, CA
K. Schuebel	IRTA Fellow	LNG, NIAAA

Objectives:

Decreased serotonin metabolism has been strongly implicated in impulsive behaviors in man and with alcohol preference in rodents. Tryptophan hydroxylase (TPH) codes for the rate-limiting enzyme in the production of serotonin. The purpose of this study is to determine the regulation of the genes coding for these proteins and their role in controlling behaviors in transgenic mice.

Methods Employed:

Cloning and gene constructions were performed by standard procedures. Sequences from mouse tryptophan hydroxylase, mouse metallothionein, firefly luciferase, HSV thymidine kinase (tk), and pUC sequences have been combined to form the appropriate vectors for the studies outlined below. Methods used include restriction endonuclease cutting, filling with Klenow enzyme, fragment isolation, ligation, site-directed mutagenesis, dideoxy sequencing, random primer labeling, polymerase chain reaction and *in situ*, northern and southern hybridization analysis.

DNA constructions were introduced into mice by injection into fertilized eggs by C. Readhead at Cedars Sinai Medical Center.

Major Findings:

Serotonin is synthesized in the raphe neurons of the brain as well as mast cells, mononuclear leucocytes,  $\beta$ -cells of the islets of Langerhans, intestinal and pancreatic enterochromaffin cells, and in the pineal gland, where it is rapidly metabolized to melatonin. TPH is found only in cells that synthesize serotonin. In the raphe neurons, TPH is the rate-limiting enzyme in the biosynthesis of serotonin. The Km of TPH is reported to be 50-120  $\mu$ M. Tryptophan, the substrate of this reaction, is present in the brain at nonsaturating levels of 30  $\mu$ M. The product of this hydroxylation, 5-hydroxytryptophan, is rapidly decarboxylated to serotonin. Hence, TPH enzyme concentration, as well as its activity, regulate serotonin production in the brain. TPH concentration is regulated by its synthesis from mRNA that is directly related to the level of TPH gene activity.

To investigate the role of TPH in regulating serotonin metabolism and role of serotonergic neurons in behavior in whole animals, transgenic mice are being made in collaboration with Dr. C. Readhead at Cedars Sinai Medical Center. Several DNA constructs have been made which are being introduced into mice. These will be assayed for function and/or behavioral effects.

In the initial set of transgenic animals the gene constructions pTL3 and pTL2 are being introduced into mice to identify the regions of the TPH gene promoter required for the control of developmental and tissue-specific expression. pTL2 and pTL3 have 511 bp and 7200 bp of the TPH promoter fused to the luciferase reporter gene, respectively. pTL3 has been injected into mice, pups have been born and are being assayed by PCR analysis for stable incorporation of the construct. The expression of pTL3 in these transgenics will be assayed for

tissue-specific and developmental expression by *in situ* and northern hybridization analysis. Luciferase expression from pTL3 will be compared with endogenous TPH gene expression in the same animal.

In the second set of transgenic mice, a construct will be introduced into mice to create a mouse in which it should be possible to specifically ablate serotonin-producing cells. The mouse genomic TPH promoter sequence has been fused to the structural gene coding for HSV tk. Initially, these mice will be assayed for tissue-specific expression of the TPH-tk fusion gene, which should be expressed in tissues normally expressing TPH. If tissue-specific expression occurs, the mice will be given the antiherpetic drug, Ganciclovir, to kill cells expressing HSV tk. This offers a novel and specific approach for the ablation of dorsal raphe neurons. Our aim is to create a mouse that is unable to synthesize serotonin and which does not contain any serotonergic cells. The effects of ablation of the serotonergic neurons on behavior will then be assessed.

Lastly, the effect of an abnormally high or low TPH content in the brain will be investigated. The metallothionein promoter has been fused to the cDNA coding for TPH. This fusion gene, when introduced into mice, should allow induction of TPH production by the addition of  $Zn^{2+}$  to the animals drinking water or by injection of  $Cd^{2+}$ . These mice should either have increased serotonin production, reduced tryptophan levels or both. Next, the metallothionein promoter is being fused to the TPH gene in an inverted orientation to yield an antisense TPH mRNA. Expression of the antisense TPH mRNA should bind endogenous TPH mRNA. This hybrid should be degraded by RNase H as well as interfere with TPH translation, thereby reducing TPH production to effect a lowering of serotonin biosynthesis. These mice may enable the switching on or off the mouse's TPH in a regulated fashion. The effects of increasing or decreasing TPH activity on serotonergic behaviors will be assessed.

#### Significance to Biomedical Research and the Program of the Institute:

TPH is the rate-limiting enzyme in the production of serotonin. Low turnover of serotonin is associated with impulsive and aggressive behaviors, increased behavioral arousal, and intolerance to delay. These behaviors are prominent features of early onset alcoholism associated with features of impulsive and antisocial behavior. It is therefore likely that the level and activity of TPH plays a major role in these behaviors. Our studies on the TPH gene have improved our understanding of the structure, function, and expression of this enzyme. We now extend these to identify developmental and tissue-specific regulatory sequences. Furthermore, the transgenic mice may reveal the origins of genetic behavioral differences.

#### Proposed Course:

To understand the effects of environmental and physiological factors, such as stress on serotonin function, as well as genetic influence on serotonergic behaviors, we are investigating the regulatory control of TPH gene expression and the role of TPH activity and serotonin in behavior. Studies to determine regions of the promoters of these genes involved in tissue-specific and developmental control of expression will be pursued. These transgenic mice provide an ideal system exploitable to study the factors influencing serotonergic behaviors.

#### Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00087-01 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on DNA Single-Strand Conformation Prediction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. Nielsen Senior Staff Fellow LNG, NIAAA

Others: D. Goldman Chief LNG, NIAAA

A. Novoradovsky Visiting Associate LNG, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Genetics

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda MD 20892

TOTAL STAFF YEARS:

.45

PROFESSIONAL:

.25

OTHER:

.20

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To identify genetic contributions to alcoholism vulnerability, we have been using single-strand conformational polymorphism (SSCP) analysis. SSCP analysis identifies polymorphic alleles through the detection of altered single-strand DNA secondary structure. The altered secondary structure occurs due to nucleotide changes in DNA sequence. To aid in our understanding of the SSCP technique, we have devised a computer program to emulate the folding of single-strand DNAs.



Project Description:Investigators:

D. Nielsen	Senior Staff Fellow	LNG, NIAAA
D. Goldman	Chief	LNG, NIAAA
A. Novoradovsky	Visiting Associate	LNG, NIAAA

Objectives:

The objective of the Section of Molecular Genetics is to understand the genetic determinants of serotonergic function and behaviors. Our studies have concentrated on genes likely to play a role in serotonergic metabolism. To identify mutations in these genes, we have been using the single-strand conformation polymorphism (SSCP) analysis technique. Regions of the genome are amplified by polymerase chain reaction, denatured, and electrophoresed in a nondenaturing gel. Upon entering the gel the single-strand DNA folds into a secondary conformation based on its DNA sequence. Mutations as small as a one nucleotide substitution can be identified. Since this technique is based on the folding of single-strand DNA, we wanted to predict single-stranded DNA structure to design the optimal PCR primers to produce altered SSCP mobilities. This ability to predict DNA structure would aid in our ability to identify mutations.

Methods Employed:

Both the Macintosh computer and Convex supercomputer were used to create and execute the DNA-Fold program. Methods used include DNA isolation, polymerase chain reaction, and autoradiography.

SSCP analysis was performed on human DNA samples. Regions of the genes were amplified with oligonucleotide primers by the polymerase chain reaction technique, denatured, and electrophoresed on nondenaturing polyacrylamide gels. Bands were visualized and analyzed by autoradiography or phosphorimaging.

Major Findings:

To predict the structure of DNA analyzed by SSCP, we have modified Michael Zuker's LRNA RNA folding program to emulate the folding of single-strand DNA molecules. We modified the energy files utilized by the LRNA and CRNA RNA folding algorithms to emulate folding of single-strand DNA. Among the changes to the energy files were those made to prevent pairing between guanine and thymine bases as occurs between guanine and uracil in RNA and the constraints on loop nucleotide sequences were removed. This is due to a methyl group on the thymine in the C-5 position of thymine. We call this program the DNA-Fold program.

To test the program, we analyzed the two polymorphic alleles of the human ALDH2 gene because we had observed that these alleles were indistinguishable by SSCP analysis.

Primers were made that either alter complementarity to an internal sequence or add a 5' tag sequence to alter the folding of the single-strand ALDH2 DNA PCR products. Differences in secondary structure of the single-strand DNA were assessed by SSCP analysis. The mobility of the DNA from the polymorphic ALDH2 alleles was compared to the predicted structures. Our results demonstrate that by using the DNA-Fold program alterations in single-strand DNA conformation may be predicted.

Significance to Biomedical Research and the Program of the Institute:

The DNA-Fold program will enhance our understanding of the folding of single-strand DNA. This will aid in the design of primers for analysis of mutations by SSCP analysis thereby allowing us to more efficiently and predictably detect alterations in genes.

Proposed Course:

The DNA-Fold program has only been rigorously evaluated for its ability to predict SSCP mobility alterations for variants of the ALDH2 gene. We will use the DNA-Fold program to predict the mobility of other DNA variants and consider modifications of the program based on the accuracy of these predictions.

Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00234-12 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Studies on Serotonergic Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. Nielsen Senior Staff Fellow LNG, NIAAA

Others: D. Goldman Chief LNG, NIAAA  
S. Hall NRC Fellow LCS, NIAAA  
W. Henley IPA LNG, NIAAA  
G. Jenkins Biologist LNG, NIAAA  
B. Nakhai Visiting Fellow LNG, NIAAA  
K. Schuebel IRTA Fellow LNG, NIAAA

COOPERATING UNITS (if any)

Program Resources Inc., Frederick, MD (M. Dean); University of Colorado, Boulder (T. Johnson)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Genetics

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda MD 20892

TOTAL STAFF YEARS:

3.55

PROFESSIONAL:

1.45

OTHER:

2.10

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To identify genetic contributions to alcoholism vulnerability, we focused on serotonergic behaviors, since a subtype of alcoholism is associated with decreased serotonin turnover. Serotonin biosynthesis is governed by tryptophan hydroxylase (TPH), which is rate-limiting. Levels of tryptophan, a precursor in serotonin biosynthesis, are modulated by tryptophan 2,3-dioxygenase (TDO). We hypothesize that genetic variants of TPH and TDO and factors controlling their gene expression play a major role in behavior. We cloned and sequenced the cDNA and gene for murine TPH gene. TDO gene expression was detected for the first time in brain. Polymorphic variants of the human, mouse, and macaque TPH were identified by SSCP analysis. The human TPH gene was mapped to chromosome 11p15.5. The human TPH polymorphism associated with CSF 5-HIAA concentration in alcoholic, impulsive, Finnish, violent offenders. The polymorphism associated with history of suicidal attempts and of multiple suicidal attempts in alcoholic, violent Finns. The polymorphic TPH alleles are being sequenced. The factors and DNA sequences controlling the expression of the mouse TPH gene are being identified. DNA regions controlling tissue-specific and constitutive expression have been identified using DNA fusion constructs. These regions are being analyzed by electrophoretic mobility shift analysis to identify transcription factors and their cognate binding sites that regulate TPH gene expression. A protein factor that represses TPH gene expression is being cloned and characterized.

Project Description:Investigators:

D. Nielsen	Senior Staff Fellow	LNG, NIAAA
M. Dean	Staff Scientist	PRI, Frederick, MD
D. Goldman	Chief	LNG, NIAAA
S. Hall	NRC Fellow	LCS, NIAAA
W. Henley	IPA	LNG, NIAAA
G. Jenkins	Biologist	LNG, NIAAA
T. Johnson	Professor	IBG, UC,
		U of CO, Boulder
B. Nakhai	Visiting Fellow	LNG, NIAAA
K. Schuebel	IRTA Fellow	LNG, NIAAA

Objectives:

The objective of the Section of Molecular Genetics is to understand the genetic determinants of serotonergic function and behaviors contributing to alcoholism. These studies attempt to identify genetic loci and variants as well as regulatory features involved in the clinical subtype of alcoholism distinguished by impulsive and aggressive behavior. Decreased serotonin metabolism has been strongly implicated in impulsive and aggressive behaviors in man and with alcohol preference in rodents. We have concentrated on genes likely to play a role in serotonergic metabolism. Since tryptophan hydroxylase (TPH) is the rate-limiting enzyme in the production of serotonin and tryptophan 2,3-dioxygenase (TDO) regulates tryptophan concentrations, we have focused on the genetic regulation of the genes coding for these proteins and their role, as well as that of natural genetic variants, in controlling behaviors in humans, monkeys, and mice.

Methods Employed:

Cloning and gene constructions were performed by standard procedures. Sequences from mouse tryptophan hydroxylase, mouse tryptophan 2,3-dioxygenase, firefly luciferase, actin, mouse metallothionein, HSV thymidine kinase, Tn5 neomycin-kanamycin resistance,  $\beta$ -galactosidase genes, as well as SV40, RSV, CMV, and MMTV promoters and regulatory elements, and pUC sequences, were combined to form the appropriate vectors for the studies outlined below. Methods used include restriction endonuclease cutting, filling with Klenow enzyme, digestion with T4 DNA polymerase, fragment isolation, ligation, site-directed mutagenesis, dideoxy DNA sequencing, random primer labeling, reverse transcription, northern hybridization analysis, southern hybridization, cDNA cloning, lambda propagation and screening, polymerase chain reaction, reverse transcriptase-polymerase chain reaction, and electrophoretic mobility shift analysis.

Serotonin synthesizing and secreting and serotonin receptor-containing cell lines were grown in tissue culture. Serotonergic cell lines include TT cells, a human medullary thyroid carcinoma cell line, Neuro-2a, a mouse neuroblastoma cell line, and P815 cells, a mouse mastocytoma cell line. Mouse 3T3 fibroblast cells serve as a nonserotonergic control. The effects of compounds on gene expression in the serotonergic cell lines and in mouse pineal glands cultured *in vitro* were assayed by northern hybridization of endogenous TPH and cyclophillin mRNA. These cell lines have been transfected with various DNA plasmid constructions using calcium phosphate or electroporation. Transiently transfected cells, as well as stable, G418 resistant cell lines have been made. Promoter function is assayed using highly sensitive enzyme assays for the reporter genes luciferase and  $\beta$ -galactosidase, as well as by northern hybridization analysis. mRNA was extracted from cell lines and tissues by the guanidinium thiocyanate-phenol-chloroform method. The RNA was electrophoresed and transferred to nylon membranes and hybridized with random-primed radiolabeled probes.



Single-strand conformation polymorphism (SSCP) analysis was performed on DNA samples of human, mouse, vervet and rhesus macaque. Regions of the tryptophan hydroxylase gene were amplified with oligonucleotide primers by polymerase chain reaction, denatured, and electrophoresed on nondenaturing polyacrylamide gels. Bands were analyzed by autoradiography or phosphorimaging. Genetic mapping, using the results of the SSCP analyses of the CEPH family database, was performed by M. Dean employing the MAPMAKER and LINKAGE programs. Association to serotonergic traits was by standard statistical analyses.

TPH antisense oligothiophosphonucleotides were synthesized on a ABI 394 DNA/RNA synthesizer. Oligos were injected into the cerebral ventricle via acutely implanted canula in anesthetized rats.

#### Major Findings:

##### Tryptophan Hydroxylase

Serotonin is synthesized in raphe neurons of the brain as well as mast cells, mononuclear leucocytes,  $\beta$ -cells of the islets of Langerhans, intestinal and pancreatic enterochromaffin cells, and in the pineal gland, where it is rapidly metabolized to melatonin. TPH is found only in cells that synthesize serotonin. In raphe neurons, TPH is the rate-limiting enzyme in the biosynthesis of serotonin. The  $K_m$  of TPH is 50-120  $\mu$ M. Tryptophan, the substrate of this reaction, is present in the brain at nonsaturating levels of 30  $\mu$ M. The product of this hydroxylation, 5-hydroxytryptophan, is rapidly decarboxylated to serotonin (5-hydroxytryptamine). Hence, TPH enzyme activity controls serotonin production in the brain. TPH enzyme activity is regulated at various levels which include gene transcription and, perhaps, mRNA stability. Hence, TPH enzyme concentration is directly related to the level of TPH gene activity.

TPH is a member of the family of aromatic amino acid monooxygenases. A full length, murine TPH cDNA was previously cloned from a mastocytoma cDNA library. The cDNA encodes a 447 amino acid protein. Northern analysis of mRNA isolated from various mastocytoma cell lines, pineal, duodenum and midbrain, revealed two TPH mRNA size classes: a 1900 base pair TPH mRNA and a minor (5-10%) 4000 base pair partially processed, precursor TPH mRNA.

The TPH cDNA was used to probe a mouse genomic library. Overlapping clones, spanning 40 kilobases of the murine gene, were isolated. The murine TPH gene is 21 kilobase in length and mapped to chromosome 7. Sequence analysis of the 5' upstream regulatory regions reveal the presence of many putative regulatory regions. Besides, conserved TATA and CCATT boxes are sequences related to the consensus binding sequences of AP-1, AP-2, and AP-3. Regulatory sites are present for cyclic AMP regulation, glucocorticoid receptor binding, the urea cycle enzyme sequence, retinoblastoma gene product control element, Spl, NF-1, NFK-B and octomer binding sites as well as general helix-turn-helix transcription factor binding sites.

TPH gene expression has been studied *in vitro* to define the mechanisms controlling its regulation. TPH gene expression initially is being studied in P815 cells. Analysis of the promoter region of the mouse TPH gene led us to analyze the effects of several compounds on TPH gene expression. Dexamethasone, 8-chlorophenyl-cAMP (8-CPT-cAMP, a cell membrane-permeable cAMP analog), and serotonin were added to P815 cells growing in culture. At various time points, RNA was isolated and TPH mRNA content was quantified by northern gel hybridization analysis. Absolute TPH mRNA content was determined using of *in vitro* SP6-synthesized TPH mRNA. Actin or cyclophilin mRNA was also quantified and used to correct for intersample variation. TPH gene expression was regulated in P815 mastocytoma cells. In unstimulated P815 cells, TPH mRNA is present at 470 molecules per cell. Addition of 8-CPT-cAMP (100  $\mu$ M) increased TPH mRNA content more than two-fold in 48 hours to 1130 transcripts per cell. After a 48 hour stimulation with dexamethasone (1  $\mu$ M), TPH mRNA content increased two-fold to 950 transcripts per cell. Serotonin (100  $\mu$ M) elicited the opposite effect.

TPH mRNA content decreased to 90 transcripts per cell, representing more than a five-fold decrease in TPH mRNA content in 48 hours. If serotonin caused a total cessation of TPH mRNA transcription, the half-life of the TPH mRNA would be 22 hours under these conditions. Hence, the expression of TPH mRNA is regulated over a twelve-fold range in 48 hours in P815 cells. We hypothesize a negative feedback-loop regulating serotonin production. In this model, serotonin down-regulates its own synthesis by down-regulating TPH gene expression. This may be mediated through the binding of serotonin to the 5-HT<sub>1A</sub> autoreceptor resulting in the inhibition of adenylyl cyclase, thereby reducing intracellular cAMP content. Since 8-CPT-cAMP increases TPH gene expression, a reduction in cAMP through the binding of serotonin to the 5-HT<sub>1A</sub> receptor may reduce TPH gene expression and, ultimately, serotonin biosynthesis. Stress, which induces glucocorticoid release, therefore may stimulate TPH gene expression.

TPH mRNA content was measured in pineal glands in collaboration with D. Klein, NIAID. When isolated pineal glands were incubated in medium containing norepinephrine, there was a 83% decrease in TPH mRNA over the course of the experiment. This effect on TPH mRNA content was not considered significant.

To analyze the plethora of possible regulatory sites, the 5' upstream region of the mouse TPH genomic clone was fused to the luciferase gene. When transfected into tissue culture cells, a fusion mRNA of 2500 bp was produced containing 98 bases of the 5' untranslated region from TPH, followed by multiple cloning site sequence, luciferase 5' untranslated region, luciferase coding sequence, and luciferase and SV40 3' untranslated regions. This transcription fusion allowed gene expression to be easily assayed with great sensitivity. This DNA is being introduced into tissue culture cells using the calcium phosphate DNA transfection method along with pSV- $\beta$ -gal in order to provide an unregulated, internal transfection control. DNAs are being transfected into several serotonin-synthesizing cell lines including P815 and Neuro-2a as well as the nonserotonin-producing NIH 3T3 cells. To identify regulatory promoter sequences, plasmid constructions were created that contain up to 7,200 bp of the TPH promoter fused to luciferase. Using these TPH promoter deletion plasmids, several regions of the TPH promoter have been delineated.

A silencer region has been identified in the TPH promoter that represses TPH expression only in cells (P815) that express large amounts of TPH mRNA. The DNA binding site of the silencer region is a seven base pair site. Electrophoretic mobility shift assays (EMSAs) are being used to identify transcription factor binding. Nuclear extracts from P815, Neuro-2a, HeLa, and NIH 3T3 cells were incubated with <sup>32</sup>P-labeled DNA fragments from the TPH promoter to detect binding of specific transcription factors. Binding was confirmed by competition with nonlabeled DNA fragments. EMSAs have demonstrated that the tissue-specific silencer region is a unique seven base pair sequence that binds a protein(s) factor. UV-crosslinking and SDS-PAGE analysis has demonstrated that this binding protein has a molecular weight of 27,000. Sizing gels and Ferguson analysis suggest that either multiple factors bind to this DNA sequence or the factor binds as a dimer. We have isolated a putative clone for this factor which we are characterizing. This clone appears to code for a unique transcription factor. Deletion analysis of the TPH-luciferase fusions has identified a second silencer region that represses TPH transcription in cells that do not express TPH. This region may control the tissue-specific expression of TPH while the region described above may regulate TPH content in cells that make TPH. Three other TPH promoter regions have been located which activate transcription. Two of these regions appear not to be tissue-specific while the third region activates TPH transcription only in cells that synthesize TPH. The TPH-luciferase fusion constructs are also being used to identify compounds regulating TPH transcription. Several compounds including dexamethasone increase TPH-luciferase expression. We are presently identifying the sites of action of this synthetic glucocorticoid and have located a negative GRE in addition to several conventional GRE sites.

Antisense oligonucleotides are being used to alter TPH expression in the brains of rats. Oligothiophosphonucleotides have been synthesized on our oligonucleotide synthesizer corresponding to the region spanning the translational initiation site of the rat TPH mRNA. These antisense oligos are being assayed for their ability to inhibit serotonin production in various regions of the brain. Oligothiophosphonucleotides are being introduced, via canula, into the ventricles of the rat brains. Initial studies have indicated that the TPH antisense oligos have the ability to reduce brain serotonin content. The effect of decreased serotonin metabolism on behavior will be assessed with the appropriate serotonergic behavioral tests.

To identify a human TPH polymorphism, DNA was amplified and assayed using the SSCP technique. A polymorphism was revealed in intron 7. Allele frequencies in Caucasians were 0.40 and 0.60. In collaboration with M. Dean (NCI), DNA samples from 24 informative CEPH families were typed for the polymorphism. Linkage analysis with respect to eight linked markers on chromosome 11 mapped TPH to 11p15 near the HBB and tyrosine hydroxylase loci. Intron 7 of the polymorphic alleles has been sequenced and contains two A to G transitions. We are presently investigating if these could alter processing or expression of the TPH gene. We have sequenced over 95% of the TPH mRNA (cDNA) and have found no linked mutations.

A collection of 50 impulsive and 83 nonimpulsive, alcoholic, violent offenders and 63 healthy volunteers were analyzed as to their TPH genotypes in collaboration with M. Virkkunen and R. Tokola (University of Helsinki). Many of the impulsive subjects had extremely low CSF 5-HIAA, providing an ideal population to detect an association of TPH genotype to function. We found a significant association of the polymorphism to CSF 5-HIAA concentration in this behaviorally extreme and relatively homogeneous impulsive group. Furthermore, the TPH polymorphism associated with history of suicidal attempts and of multiple suicidal attempts in the alcoholic, violent Finns. Further studies are being conducted in collaboration with L. Handelsman at the Ross VAMC. TPH genotype is being determined in substance abusers in an effort to identify additional behaviors associated with the TPH genetic variants as well as new TPH polymorphic alleles. In collaboration with J. Gelernter (Yale University), TPH genotype is being analyzed in a cohort of suicidal subjects. In collaboration with M. Asberg (Karolinska Hospital) and L. Traskman-Benz (University of Lund), TPH genotype is being determined in a group of depressed, suicidal subjects and Swedish controls.

An SSCP polymorphism has been identified in intron 8 of murine TPH. In collaboration with T. Johnson (U of Colorado), genotypes of LS X SS recombinant inbred mouse strains were determined. All but one of the alcohol sensitive (long sleep, LS) strains had the same genotype. The alcohol insensitive (short sleep, SS) strains were varied in TPH genotype. The TPH polymorphism was sequenced and shown to contain a highly repetitive sequence. Since this study began quantitative trait locus analysis of the LS X SS mice at the U of Colorado has demonstrated no association of TPH with sleep time.

In both vervet and rhesus macaque monkeys, SSCP polymorphisms have been identified in an intron of the TPH gene. These are both being correlated with serotonergic metabolism and behaviors in these monkeys.

#### Tryptophan 2,3-dioxygenase

Tryptophan availability in the brain is rate-limiting for serotonin biosynthesis. This may be controlled, in part, by the activity of TDO in the liver. TDO is the rate-limiting enzyme in the catabolism of tryptophan to kynurenine. The concentration of free tryptophan in the body is maintained relatively constant at 30  $\mu$ M. Dietary intake of tryptophan increases after a high protein meal. Ingested tryptophan initially passes through the liver via the portal vein. Since TDO has a  $K_m$  of 250  $\mu$ M, the metabolism of tryptophan occurs at a relatively low rate. But the concentration of tryptophan in the brain remains constant, even after a high protein meal, and serotonin levels remain unchanged.



To determine factors controlling brain tryptophan concentration, the expression of TDO in brain was investigated. RNA was isolated from rat brain and liver and subjected to reverse transcriptase and polymerase chain reaction with primers based on the rat TDO sequence. TDO PCR products were purified and sequenced. Identical sequences were obtained from brain and liver. TDO gene expression was detected in brainstem, cortex, cerebellum, and hypothalamus. This indicates that TDO may function in the brain to regulate tryptophan concentration.

#### Significance to Biomedical Research and the Program of the Institute:

Low turnover of serotonin is associated with impulsive and aggressive behaviors, increased behavioral arousal, and intolerance to delay. These behaviors are prominent features of early onset alcoholism associated with features of antisocial behavior. It is therefore likely that the level and activity of tryptophan hydroxylase, the rate-limiting enzyme in the production of serotonin, and tryptophan 2,3-dioxygenase, a regulator of tryptophan concentration, may play major roles in the development of these behaviors. Our studies on these genes have improved our understanding of their structure, function, and expression. Understanding the factors regulating TPH gene expression may lead to the development of new therapeutic approaches for the treatment of alcoholism. We are presently investigating the mechanisms governing their expression. Furthermore, the polymorphic TPH alleles may identify additional genetic associations that will reveal the cause of genetic behavioral differences in humans, monkeys, and mice.

#### Proposed Course:

To understand the effects of environmental and physiological factors, such as stress, on serotonin function, as well as genetic influence on serotonergic behaviors, the regulatory mechanisms of TPH and TDO gene expression are being investigated. Using transfected tissue culture cells, factors controlling TPH and gene expression and tissue-specific expression are being delineated. The studies are revealing regions in the TPH promoter involved in repression and activation of transcription by both tissue-specific and tissue-independent mechanisms. These sites will be further characterized. We may have cloned one new transcription factor that represses TPH transcription in a tissue-specific manner. This cDNA clone will be characterized and the gene for this cDNA will be cloned. If this proves to be an interesting factor, we will investigate its expression. Experiments will also begin to disrupt the binding of this factor to up-regulate TPH expression. Perhaps this may lead to development of a new therapeutic for raising brain serotonin content. The other TPH controlling sites identified will be further analyzed to identify their cognate binding factors. The TPH polymorphisms identified in humans, monkeys, and mice are being sequenced and characterized. These polymorphic genes will be analyzed to determine how they vary in function and, possibly, how they modify behavior. The human TPH polymorphism will be genotyped in more populations to determine if the association to CSF 5-HIAA and suicidal behavior can be replicated. Efforts will be made to sequence the human TPH promoter from subjects to identify alterations that made modulate TPH expression.

#### Publications:

Nielsen DA, Goldman D, Virkkunen M, Tokola R, Rawlings R, Linnoila M. Suicidality and 5-hydroxyindoleacetic acid concentration associated with a tryptophan hydroxylase polymorphism, Arch Gen Psychiatry 1994;51:34-38.

Linnoila M, Virkkunen M, George T, Eckardt M, Higley JD, Nielsen D, Goldman D. Serotonin, violent behavior and alcohol. In: Jansson B, J m ll H, Rydberg U, Terenius L, Vallee BL, eds. Towards a molecular basis of alcohol use and abuse. Basel, Switzerland: Birkh user Verlag, 1994;155-163.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00012-02 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Modulation of Anxiety by Oxytocin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. Brooks Senior Staff Fellow LNG, NIAAA

Other: D. Goldman Chief LNG, NIAAA

COOPERATING UNITS (if any)

University of Maryland (M. McCarthy)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Neurobiology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.1

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The neuropeptide oxytocin has been implicated in the control of numerous affiliative behaviors including maternal, sexual, and social behaviors. Stress is also a stimulus for the release of oxytocin. It was hypothesized that oxytocin acts in the brain to facilitate social interactions, whether they be parent/offspring, mating partners or conspecifics, by reducing anxiety during these stressful encounters. Many of these behaviors are normally expressed under restricted endocrine conditions in rodents (i.e., recently parturient, estrous, etc.) and steroids have been shown to increase oxytocin binding to its receptor in specific brain regions. Therefore, we tested if oxytocin would act as an anxiolytic in the plus-maze and the hole-board apparatus and if steroids would modulate this effect.



Project Description:Investigators:

P. Brooks	Senior Staff Fellow	LNG, NIAAA
D. Goldman	Chief	LNG, NIAAA
M. McCarthy	Assistant Professor	U of Maryland

Objectives:

The neuropeptide oxytocin has been implicated in the control of numerous affiliative behaviors, including maternal, sexual, and social behaviors. Stress is also a stimulus for the release of oxytocin. It was hypothesized that oxytocin acts in the brain to facilitate social interactions, whether they be parent/offspring, mating partners or conspecifics, by reducing anxiety during these stressful encounters. Many of these behaviors are normally expressed under restricted endocrine conditions in rodents (i.e., recently parturient, estrous, etc.) and steroids have been shown to increase oxytocin binding to its receptor in specific brain regions. Therefore, we tested if oxytocin would act as an anxiolytic in the plus-maze and hole-board apparatus and if steroids would modulate this effect.

Methods Employed:Experiment One

NCI Swiss mice males were left gonadally intact and females were ovariectomized and injected subcutaneously with either B-estradiol (5 ug) in sesame oil or vehicle for two days prior to behavioral testing. On the day of behavioral testing, animals were injected intraperitoneally with oxytocin or vehicle and testing commenced 40 minutes later. A dose response curve indicated that 3ug/kg was the optimal dose.

Experiment Two

Female swiss mice were cannulated into the lateral ventricle using PE-20 tubing. After four-five days of recovery, females were either treated with estrogen or vehicle as before and on the third day were infused into the ventricle with either oxytocin (8 ug), the specific oxytocin antagonist, ornithine vasotocin (8 ug), or vehicle (saline) in a 4 ul volume. Twenty minutes after infusion, they were tested for anxiety.

Behavioral testing was conducted using an elevated plus-maze and an electronically monitored hole-board. Data were collected by computer on an observer operated activity monitor.

Following the completion of behavioral testing, brain were removed and frozen at -70°C. Cryostat sections were taken through the hypothalamus and amygdala, and processed for oxytocin receptor autoradiography.

Major Findings:Experiment One

In males, oxytocin induced a mild but significant increase in parameters indicative of an anxiolytic action. In ovariectomized females, measures of anxiety were higher in general than in males. Estrogen alone did not change measures of anxiety but when combined with oxytocin there was a significant anxiolytic effect; in females without estrogen, there was no effect of oxytocin, suggesting that estrogen had facilitated the ability of oxytocin to reduce anxiety.

Experiment Two

There was again a significant anxiolytic effect of oxytocin but only in the estrogen-treated females and, most notably, there was an increase in anxiety in females receiving the antagonist, but only in those treated with estrogen. When females received no estrogen, there was no difference between those infused with oxytocin versus antagonist. There was no evidence of adverse motor effects by any of the treatments although oxytocin frequently induced a high level of grooming behavior which may have interfered with other behavioral effects.

Analysis of receptor binding data obtained to date indicates that estrogen increases oxytocin receptor binding in the septal area. Other brain areas are now being analyzed.

Significance to Biomedical Research and the Program of the Institute:

Alcoholism clusters with anxiety disorders and antisocial personality in both the individual and in families. Alcoholism is also across cultures more prevalent in males. Sex-influenced factors for affiliative behaviors and anxiety are thus of importance for a full understanding of the causes of alcoholism and related disorders.

Proposed Course:

The genetic typing will begin this fall and analysis will follow shortly. We expect results by next year.

Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AA 00013-02 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification of Novel mRNAs Synthesized During Brain Sexual Differentiation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. Brooks Senior Staff Fellow LNG, NIAAA

Others: S. Akhter IRTA Fellow LNG, NIAAA  
D. Goldman Chief LNG, NIAAA

COOPERATING UNITS (if any)

University of Maryland (M. McCarthy)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Neurobiology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.1

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The process of sexual differentiation of the brain into a male or female type occurs during the early postnatal period in rodents. This process is critically dependent upon the steroid hormones testosterone and estrogen. Despite knowledge of the major factors involved in brain sexual differentiation, little is known about the molecular mechanisms involved. To address this question, we plan to use the technique of differential display to identify cDNAs synthesized during brain sexual differentiation, in particular, cDNAs which are different in the male vs. female brain, and cDNAs that are stimulated by androgen treatment of female animals. The differential display technique involves amplification of cDNAs corresponding to mRNAs from two different samples. The amplified cDNAs are run on a gel, and bands that differ between the two samples are then identified. Differentially expressed cDNAs will be sequenced and patterns of expression determined by in situ hybridization. Antisense techniques will then be used to block the translation of differentially expressed mRNAs, followed by morphological and functional assessment of sexual differentiation.

Project Description:Investigators:

P. Brooks	Senior Staff Fellow	LNG, NIAAA
S. Akhter	IRTA Fellow	LNG, NIAAA
D. Goldman	Chief	LNG, NIAAA
M. McCarthy	Assistant Professor	U of Maryland

Objectives:

The major objective of this project is to identify novel gene transcripts which are synthesized in the neonatal brain during sexual differentiation, and transcripts which are regulated by steroid hormones in the neonatal brain. Such transcripts may encode proteins important for sexual differentiation of the brain.

Methods Employed:

The differential display technique (described in Z01 AA 00084-01 LNG) will be used. Total RNA will be isolated from the brains of female and male newborn rats, or female newborns treated with testosterone during the critical period of sexual differentiation. RNA will be reverse transcribed and analyzed using differential display to search for cDNAs that present only in females or only males, or cDNA which are stimulated by testosterone treatment in the neonatal period. Tissues to be examined include areas which are classically recognized to be sexually differentiated, such as the hypothalamus, and also brain regions involved in cognitive processes, such as the cerebral cortex and hippocampus.

Major Findings:

This project is in the beginning stages.

Significance to Biomedical Research and the Program of the Institute:

Across cultures, alcoholism is two-three times more prevalent in males than females. Also, the prevalence of several psychiatric conditions (e.g., unipolar depression) which show co-morbidity with alcoholism differs between males and females. Thus, further understanding of the molecular mechanisms of sexual differentiation and identification of sexually dimorphic transcripts in brain areas involved in cognitive processes will be important for a complete understanding of the neurobiological mechanisms underlying alcoholism and associated psychopathologies.

Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00083-01 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Expression and Regulation of DNA Methyltransferase in the Mammalian Brain

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. Brooks Senior Staff Fellow LNG, NIAAA

Others: D. Goldman Chief LNG, NIAAA

C. Marietta Physiologist LNG, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Neurobiology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0.8

PROFESSIONAL:

0.4

OTHER:

0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

DNA methylation is critically involved in the control of chromatin structure and gene expression. There are data in the literature suggesting that alcohol may influence the pattern of DNA methylation. Effects of alcohol on DNA methylation would be important for understanding the teratogenic and carcinogenic effects of alcohol and its metabolites, as well as for identifying and understanding long-term effects of alcohol on gene expression in the brain. Therefore, we have begun a series of studies on the localization and expression of DNA methyltransferase (MTase), the enzyme responsible for DNA methylation. Using reverse transcriptase-PCR, a cDNA for MTase was cloned and sequenced for use in mRNA measurements. Northern blot and RNase protection assays were used to detect MTase mRNA in various regions of the brain and other mouse tissues. In addition, an assay for MTase enzymatic activity using whole cell extracts has been developed. MTase mRNA and enzyme levels will be assessed in different brain regions, during development and aging, and the effects of ethanol and its metabolite acetaldehyde on MTase will be determined.

DNA methylation is thought to influence gene expression via nuclear protein binding to methylated DNA. Several methyl-DNA binding proteins have been identified. One of these, MeCP-BP2, is relatively abundant in brain. The distribution and regulation of MeCP-BP2 will be studied in parallel with MTase.

Project Description:Investigators:

P. Brooks	Senior Staff Fellow	LNG, NIAAA
D. Goldman	Chief	LNG, NIAAA
C. Marietta	Physiologist	LNG, NIAAA

Objectives:

The major objectives of this project are to: (1) identify sites of DNA methyltransferase (MTase) expression in the mammalian brain and peripheral tissues; (2) examine the regulation of MTase during brain development and during aging; (3) study the regulation of MTase by ethanol and its major metabolite, acetaldehyde; (4) study the regulation of MTase activity by steroid hormones in the brain and peripheral tissues; (5) map the distribution of Methyl CpG binding protein (MeCPBP-2) expression in brain; and (6) search for functional variants of the human MTase gene.

Methods Employed:

The reverse transcriptase-PCR procedure is used to obtain a mouse cDNA for MTase. The cDNA was sequenced by Cycle Sequencing. Northern blot and RNase protection assays are used to detect MTase mRNA in different mouse tissues. *In situ* hybridization and immunocytochemistry are being used to localize the enzyme to specific regions and cells in the brain. For measurement of enzymatic activity, the procedure of Li et al. (1992) has been adapted for adult tissue samples. In this assay, whole cell extracts are made by extraction with buffer containing 0.4 M NaCl. Endogenous nucleic acids are removed by adding 1 volume of a 50% slurry of DEAE Sephacel in Tris buffer. After centrifugation, the supernatant is used for MTase assay, in which the incorporation of tritiated S-Adenosyl Methionine (the methyl donor) into poly dI-dC is measured.

Major Findings:Peripheral Tissue Distribution of MTase mRNA in Rats and Mice

Very low levels of MTase mRNA are found in the adult rodent liver. Higher levels are present in the kidney, testis, ovary, brain, and spleen. The thymus has the highest levels of MTase mRNA of any tissue examined.

MTase in Brain

Surprisingly, the brain has relatively high levels of MTase mRNA. Measurements in different brain regions show lowest levels in brainstem, higher levels in cortex, hippocampus, and striatum, and highest levels in cerebellum and olfactory bulb.

Developmental Regulation

MTase mRNA is present at higher levels in newborn (postnatal day two) liver and brain than in the corresponding adult tissues, indicating down-regulation during development. In the brain, MTase mRNA decreases from embryonic day 16 to postnatal day 19, then remains constant through adulthood. More detailed developmental studies are now underway.

MTase Enzymatic Activity

An assay for MTase enzymatic activity has also been developed, based on the procedure of Li et al. (1992). MTase activity in tissue extracts parallels the mRNA levels, indicating that the mRNA measurements we have performed may be reflective of enzymatic activity.

MeCP-BP2

A cDNA encoding MeCP-BP-2 was obtained from mouse brain, and the MeCP2 cDNA is presently being sequenced.

Significance to Biomedical Research and the Program of the Institute:

The significance of DNA MTase in the brain is presently unknown. In other systems, MTase activity is closely tied to DNA synthesis. Neurons in the adult central nervous system are clearly not undergoing cell division (with the exception of the olfactory bulb and the dentate gyrus of the hippocampus). Thus, if MTase is present in neurons, as the data indicate, it must have an additional function separate from DNA synthesis. MTase in the brain could be crucial for many functions, including the regulation of gene expression, DNA repair, and a role in learning and memory.

DNA methylation is critically involved in chromatin structure and the control of gene expression. Alterations in DNA methylation pattern would be expected to result in aberrant gene expression, with corresponding phenotypic effects such as developmental abnormalities and cancer. Thus, this research could have broad implications for helping to understand long-term behavioral and cognitive changes in alcoholics, damage to the brain and other organs, alcohol-induced carcinogenesis, and both fetal alcohol syndrome and fetal alcohol effects.

Steroid hormones have profound effects on peripheral target tissues as well as the brain. Sex steroid effects are responsible for sexual differentiation of the brain, which is of relevance to alcohol research since alcoholism is two-three times more prevalent in males than females. Effects of steroids on MTase activity in the developing brain could be involved in brain sexual differentiation, with long-term effects on adult behavior. Since steroids have profound effects on the adult brain, steroid effects on MTase may be important for the regulation of behaviorally important genes in the adult brain as well.

Proposed Course:

We will continue developmental studies on MTase mRNA and protein in brain. We will study the distribution of MTase mRNA (and protein, if possible) in human brain samples. We will study possible effects of steroid hormones in MTase activity in the developing and adult brain. We will examine possible effects of ethanol and acetaldehyde on MTase activity in rodents, as well as in tissue culture models of neuronal differentiation (PC-12 cells). We will examine the effects of blocking MTase with antisense oligonucleotides to DNA MTase and MeCP-BP-2 on methylation patterns and genomic imprinting of specific genes. We will search for genetic variants of MTase in human disease states.

Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00084-01 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic and Neurobiological Factors in Ethanol Sensitivity and Korsakoff Syndrome

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. Brooks Senior Staff Fellow LNG, NIAAA

Others: S. Akhter IRTA Fellow LNG, NIAAA  
D. Goldman Chief LNG, NIAAA  
C. Marietta Physiologist LNG, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Neurobiology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0.4

PROFESSIONAL:

0.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Within the Laboratory of Neurogenetics, strategies being used to search for genetic variants underlying vulnerability to alcoholism and associated psychopathologies include analysis of candidate genes and linkage analysis. Recently, a novel approach was developed for identifying polymorphic genetic markers and genomic regions which are associated with a specific trait. This technique, known as random amplification polymorphic detection (RAPD), involves using PCR with individual 10 base oligonucleotide primers. Using these 10-mers, each DNA amplification generates 1-10 discrete bands on an agarose gel. A proportion of these bands are polymorphic and can be used as genetic markers, which are in linkage or association with a trait. Markers of interest can then be mapped to specific chromosomal regions.

A particularly valuable use of RAPD involves analysis of congenic animals bred for a specific trait. With congenic animals, relatively few polymorphisms will be identified, but those that are found have a greater chance of being associated with the trait of interest. We will utilize the RAPD technique in collaboration with Dr. Bruce Dudek, who has developed a congenic line of mice selected for high sensitivity to alcohol-induced activity.

A variation of the RAPD procedure, known as differential display, is being used to study the expression of genes which are differentially expressed in different brain regions. In this procedure, RNA molecules are reverse transcribed using oligo dT primers, then amplified with random 10-mers. Differences in the band pattern between tissues or treatment groups can then be further analyzed. This procedure will be used to identify genes expressed in brain regions known to be susceptible to damage during thiamin deficiency, the causative agent in alcoholic Korsakoff syndrome.



Project Description:Investigators:

P. Brooks	Senior Staff Fellow	LNG, NIAAA
S. Akhter	IRTA Fellow	LNG, NIAAA
D. Goldman	Chief	LNG, NIAAA
C. Marietta	Physiologist	LNG, NIAAA

Objectives:

The major objectives of this project are the: (1) development of the random amplification polymorphism detection (RAPD) as a reproducible and reliable analytical procedure for use with mammalian genomic DNA; (2) identification of regions of the genome associated with sensitivity to ethanol-induced behavioral activation in congenic mouse lines; and (3) search for mRNAs expressed in specific brain regions susceptible to degeneration from thiamin deficiency, the cause of Korsakoff syndrome which affects a proportion of alcoholics.

Methods Employed:

RAPD is carried out on mouse or human genomic DNA as described (Nucleic Acids Res 1990;18:6531).

Rodent brain regions are dissected out and RNA isolated using the guanidine isothiocyanate phenol-chloroform extraction procedure. Differential display is carried out as described by Liang and Pardee (Current Protocols in Molecular Biology Supplement 1994).

Major Findings:

This project has been initiated very recently. However, it is clear that the RAPD technique can identify polymorphisms in genomic DNA from different mouse strains, and between humans as well. Reproducibility of the technique is a critical issue and remains to be addressed.

Significance to Biomedical Research and the Program of the Institute:

Several rodent genetic models of alcohol effects are now available. These include mouse strains which have been bred for traits related to alcohol consumption or withdrawal, or for behavioral effects of alcohol administration. Though none of these strains completely models the complexity of human alcohol abuse, such animals can be used to identify regions of the genome associated with specific traits which may be related to alcoholism. Analysis of genomic DNA from congenic mouse strains bred for sensitivity to ethanol-induced activation, using the RAPD approach, should result in identification of genomic regions related to sensitivity to alcohol-induced activity. Once such regions of the genome are identified, we could then focus on syntenic regions of the human genome in studies on human alcoholic populations. Thus, this approach may be of relevance in understanding genetic differences in vulnerability to alcoholism in humans.

Patients with Korsakoff syndrome have characteristic lesions of specific regions of the brain. The lesioned areas include the mammillary bodies, specific thalamic nuclei, and the cerebellar vermis. The damage is now known to be the result of thiamin deficiency secondary to the malnutrition which occurs in severe alcoholism. The reason why these brain areas are selectively susceptible to damage from thiamin deficiency is unknown at present. To address this issue, we plan to use the differential display approach to search for cDNAs which are expressed in brain areas susceptible to thiamin deficiency but not in other brain areas. Identification of such cDNAs should help to clarify how thiamin deficiency produces brain damage, and to increase basic knowledge of brain function.

Proposed Course:

We will continue development of the RAPD technique to ensure that it is reproducible and reliable, focusing on human and mouse DNA. We will use the RAPD approach to search for genetic differences associated with sensitivity to ethanol-induced activity in a congenic mouse line which has been bred for this trait by Dr. Bruce Dudek. Congenic lines are especially suitable for RAPD analysis, since genetic variants should be less abundant, and those that are found are more likely to be associated with the trait of interest. When such variants are found, we will map chromosomal locations to identify genomic clusters and nearby candidate genes.

Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00009-02 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Mechanisms of Drug Tolerance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Lin Visiting Fellow LNG, NIAAA

Others: D. Goldman Chief LNG, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Neurobiology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Dr. Lin has left the Institute to take a position in Taiwan, where she is continuing work on this project. The project is terminated to avoid duplication with Dr. Lin's work.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00010-02 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of the GABA-A Receptor  $\alpha 6$  Subunit in Alcohol-Induced Motor Impairment

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Lin Visiting Fellow LNG, NIAAA

Others: D. Goldman Chief LNG, NIAAA

COOPERATING UNITS (if any)

University of Maryland (M. McCarthy)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Neurobiology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Principal Investigator, Dr. Lin, has left the Institute to take a position in Taiwan, where she is continuing work on this project. The project is being terminated here to avoid duplication with her work.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00011-02 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Antisense Oligonucleotides to Block Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. McCarthy Visiting Scientist LNG, NIAAA

Others: D. Goldman Chief LNG, NIAAA  
C. McDonald Chemist LNG, NIAAA

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Molecular Neurobiology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated since a similar project is being conducted by Dr. Nielsen in the Section of Molecular Genetics, Z01 AA 00234-12 LNG.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00014-02 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic Studies on Dopamine Receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M. Adamson Senior Clinical Investigator LNG, NIAAA

Others: D. Goldman Chief LNG, NIAAA  
J. Higley Senior Staff Fellow LCS, NIAAA  
M. Linnoila Scientific Director NIAAA  
R. Robin Senior Staff Fellow LNG, NIAAA

COOPERATING UNITS (if any)

Clarke Institute of Psychiatry, U of Toronto (J. Kennedy); LVC, NCI (S. O'Brien)  
Program Resources Inc., Frederick, MD (M. Dean); U of Helsinki (R. Tokola, M. Virkkunen)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Human Neurogenetics

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A unique, highly polymorphic dopamine receptor gene, DRD4, has been identified, cloned, and sequenced. DRD4 has eight known alleles, each varying in size by a repeating motif of 48 base pairs in Exon III. Each repeating motif adds 16 amino acids to the intracytoplasmic loop, which is responsible for binding the G-protein. The DRD4.7 (seven repeat) allele has altered ligand affinity and salt sensitivity. DRD4 is an intriguing candidate for genetic variations in dopamine-related behaviors, such as reward seeking behaviors and movement disorders. Another potential genetic determinant of variations in dopamine-related behaviors is the DRD3 dopamine receptor gene, which is highly expressed in the limbic system and inhibited by most major neuroleptics effective in the treatment of mental disorders.

We estimated DRD4 and DRD3 allele frequencies in a well characterized population of Finnish alcoholics and controls. DRD4 alleles were also estimated in four other populations: Blacks, Pima Indians, Cheyenne Indians, and Jemez-Pueblo Indians, and in fourteen nonhuman primate species. Many of the Finnish alcoholics were of the early onset, impulsive type. No association between particular DRD4 or DRD3 alleles or genotypes and alcoholism was observed, nor was there an association with CSF homovanillic acid, an indicator of central dopaminergic function. Inter-population variation in frequencies of DRD4 alleles and genotypes was found, and interspecies variation in the range of alleles and degree of polymorphism were revealed for the first time.

Use of special electrophoretic techniques to detect sequence variation among the repeating 48 bp segments of the DRD4 dopamine receptor may elucidate whether the population differences in alleles and genotypes, found among the human and nonhuman primates, are behaviorally significant.

This project was previously titled, "Genetic Studies on the Dopamine D4 Receptor".



Project Description:Investigators:

M. Adamson	Senior Clinical Investigator	LNG, NIAAA
M. Dean	Staff Scientist	PRI, Frederick, MD
D. Goldman	Chief	LNG, NIAAA
D. Higley	Senior Staff Fellow	LCS, NIAAA
J. Kennedy		Clarke Institute of Psychiatry, U of Toronto, Ontario
M. Linnoila	Scientific Director	NIAAA
S. O'Brien	Chief	LVC, NCI
R. Robin	Senior Staff Fellow	LNG, NIAAA
R. Tokola	Staff Physician	U of Helsinki, Finland
M. Virkkunen	Senior Lecturer	U of Helsinki, Finland

Objectives:

The advent of new techniques for molecular genetic analysis has precipitated a wide ranging search for the "gene" for alcoholism. For example, DRD2 was proclaimed to be such a gene. Genes involved in the dopamine pathway are good candidates for alcoholism vulnerability, as dopamine plays a crucial role in reinforcement and reward seeking behavior. The DRD2 population association has, overall, not been replicated outside of the group originally reporting the finding and this association could have arisen due to population differences in marker frequency (see project number Z01 AA 00282-05 LNG).

Recently, the DRD4 dopamine receptor was discovered and cloned. DRD4 is the third member of the D2 class of dopamine receptors. D2 receptors are G protein-coupled receptors which are similar in their structure and sensitivity to neuroleptic drugs, with some important differences. In particular, the DRD4 binds the atypical neuroleptic, clozapine, with 10 times the affinity of other D2 receptors. Furthermore, the DRD4 gene is highly polymorphic, with eight known alleles, each varying in size by repeating motifs of 48 base pairs within Exon III. Each repeating motif adds 16 amino acids to the intracytoplasmic loop. The intracytoplasmic portion of the receptor is important in the binding of G-protein, and the increase in size of the intracytoplasmic loop influences the functioning of the receptor. The DRD4.7 receptor bind has been reported to spiperone with greater affinity than DRD4.4 or DRD4.2 receptors and is unaffected by the absence of salt, as are the shorter variants. Several populations of schizophrenics have been investigated in the search for an association of a variant in behavior with this polymorphic gene. Thus far, the results of these searches have been negative. This interesting gene therefore exhibits a functional polymorphism without a corresponding phenotype.

The DRD3 dopamine receptor, also of the D2 class of dopamine receptors, may also be a potential determinant of behavior. DRD3 receptor density is high in the limbic system, an area of the brain which is important in the control of cognitive, emotional, and reward processes. This increases the likelihood that it is involved in the pathogenesis or treatment response of mental disorders. Furthermore, this receptor is recognized by all major neuroleptics, including the "atypical" neuroleptics, currently used in clinical practice. A point mutation in the coding sequence of the DRD3 dopamine receptor creates a restriction site for the endonuclease Bal I and its isoenzyme MSC I. The presence of this mutation could disturb the integrity of the D3 dopamine receptor protein in the cell membrane. This variation in sequence results in two allelic forms, one with and one without a restriction site, which can be easily studied by PCR. Studies for an association with schizophrenia and bipolar illness generally have been negative.

Because of the role of dopamine in reinforcement and behavioral activation, alcoholism is a behavioral phenotype which might be associated with a DRD4 or a DRD3 variant. In two separate studies, we are comparing DRD4 or DRD3 alleles in alcoholics and ethnically matched controls and have sought a relationship between DRD4 or DRD3 genotypes and HVA level.

DRD4 alleles were also compared in several different primate species, which range in evolutionary proximity to humans. The results may afford a better understanding of the role of DRD4 in behavior.

#### Methods Employed:

Association studies in human populations: large samples of Finns, Pima Indians, Cheyenne Indians, Jemez-Pueblo Indians, and Blacks, family members and controls received comprehensive clinical psychiatric and psychological evaluations, including the SADS-L psychiatric interview, life history of impulsive behavior, the MAST for alcohol and drug use, a structured family history, and several psychological tests including the MMPI and EPQ (Eysenck Personality Questionnaire). Consensus clinical diagnoses had been made for alcoholics and controls by two raters blind to subject identities and incorporating corroborative data from relatives and from other records. A group of Finnish alcoholics and Finnish controls had been sampled for CSF and concentrations of homovanillic acid (HVA), a major dopamine metabolite, and 5-hydroxyindolacetic acid (5-HIAA), a major serotonin metabolite, were available. These monoamine metabolites had been measured by HPLC with electrochemical detection.

DRD4 allele frequencies were determined and compared in fourteen different nonhuman primate species: chimpanzee N=2, pygmy chimpanzee N=2, gorilla N=4, siamang N=2, Gelada baboon N=1, gibbon N=1, orangutan (Bornean and Sumatran) N=62, spider monkey N=4, owl monkey N=1, Colobus monkey N=1, Patas monkey N=1, ruffed lemur N=1, rhesus macaque N=8, and vervet monkey N=28.

DNA had been extracted from lymphoblastoid cell lines previously established for these species. The primate DNA were gifts from Drs. Steve O'Brien and D. Higley.

Polymerase chain reaction (PCR) was used to detect and amplify the highly guanine-cytosine rich, polymorphic region of the dopamine D4 receptor gene from genomic DNA, and PCR followed by MSC I endonuclease restriction was used to detect and amplify the DRD3 receptor gene from genomic DNA. DRD4 or DRD3 receptor gene alleles were identified on 3.5% agarose gels.

#### Major Findings:

Comparison of 113 Finnish alcoholics to 113 ethnically matched, psychiatrically interviewed controls revealed no association of a DRD4 or DRD3 allele or genotype with alcoholism. Nor was there an association with cerebrospinal fluid monoamine metabolites, HVA or 5-HIAA, both of which previously have been shown to be lower in these Finnish alcoholics.

DRD4 allele frequencies were similar to those found in a population of 150 Caucasians and reported by Kennedy et al. However, other populations exhibited differences in DRD4 allele frequencies. Blacks, Pima Indians, and Cheyenne Indians showed less variability and had lower frequencies of shorter repeat alleles. For all populations, DRD4.4 and DRD4.7 were the most abundant. DRD4.7 is the allele which has exhibited altered ligand affinity and salt sensitivity, and which may therefore differ functionally from other DRD4 alleles.

The DRD3 allele sequence without a restriction site was more frequently present in both the 113 Finnish alcoholic subjects and the 113 ethnically matched controls. The allelic frequencies were similar to those published in the literature.

The degree of DRD4 polymorphism and the frequencies of the different DRD4 alleles varied significantly among the primate species. DNA from nonhuman primate species were less diverse, with some species possessing only one or two alleles. For example, rhesus macaque monkeys were monomorphic. The only gibbon we analyzed was homozygous for the 9 repeat allele. Thus far, this allele, the 9 repeat, has not been observed in the human. The two most abundant alleles in the human, the 4 and the 7 repeat alleles, were usually not present in the other primates.

#### Significance to Biomedical Research and the Program of the Institute:

Dopamine has a crucial role in reward processes, in drug and alcohol self-administration, and in the activating effects of ethanol. The DRD4 polymorphism is not merely a genetic marker but may alter the function of this dopamine receptor. Because DRD4 may be functionally polymorphic and DRD4 is highly variable, the DRD4 dopamine receptor is very important to investigate as a genetic vulnerability factor in alcoholism. An understanding of DRD4 alleles in primate evolution may contribute to better understanding of its function. Similarly, the DRD3 dopamine receptor is an important candidate gene in alcoholism since the DRD3 receptor is located in a critical area for behavior control, binds all major neuroleptics, and is a polymorphism which may alter the function of the D3 receptor.

#### Proposed Course:

We will publish our DRD4 and DRD3 disease association and population frequency results.

The polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) approach, a technique that detects sequence variants in size or shape of the DNA fragment, will be used to further analyze different repeating segments of DRD4 alleles for sequence heterogeneity of alleles of the same repeat length. Sequencing of the individual DRD4 repeat alleles in different species may help to determine the ancestral DRD4 repeat length. DRD4 variants will also be typed in rodent and in more primate models.

Dopamine receptors DRD1 and DRD5 will also be investigated for association with alcoholism using the Finnish population of controls and alcoholics.

#### Publications:

Adamson MD, Kennedy J, Petronis A, Dean M, Virkkunen M, Linnoila M, Goldman D. DRD4 dopamine receptor alleles and CSF HVA and 5-HIAA, Neuropsychiatric Genetics, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00015-02 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Detection of Point Mutations Using Fluorescence-Based SSCP (F-SSCP)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Ellison Chemist LNG, NIAAA

Others: D. Goldman Chief LNG, NIAAA

COOPERATING UNITS (if any)

Program Resources Inc., Frederick, MD (M. Dean)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Human Neurogenetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been discontinued because it is subsumed under project Z01 AA 00290-04 LNG.

Publications:

Ellison J, Dean M, Goldman D. Efficacy of fluorescence-based PCR-SSCP for detection of point mutations, Biotechniques 1993;684-91.

Ellison J, Squires, G, Goldman D. Detection of mutations and polymorphisms using fluorescence-based dideoxy fingerprinting (F-ddF0), Biotechniques, in press.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00280-05 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetic Studies of the Electroencephalogram and Event-Related Potentials

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. Goldman	Chief	LNG, NIAAA
Others:	M. Adamson	Senior Clinical Investigator	LNG, NIAAA
	L. Brown	Clinical Director	LCS, NIAAA
	E. Davis	Psychologist	LCS, NIAAA
	M. Eckardt	Senior Investigator	LCS, NIAAA
	C. Harris	Psychologist	LNG, NIAAA
	D. Hommer	Section Chief	LCS, NIAAA
	K. White	IRTA Fellow	LNG, NIAAA

COOPERATING UNITS (if any)

Washington University (J. Rohrbaugh); London, England (M. Enoch); University of Nebraska (R. Ellingson); Program Resources Inc., Frederick, MD (M. Dean)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Human Neurogenetics

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.2

PROFESSIONAL:

1.0

OTHER:

2.2

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goal is to identify genes determining genetic variants of the electroencephalogram (EEG) and evoked responses of the EEG and event-related potentials (ERPs) to identify vulnerability genes for alcoholism and related behaviors with genetic components. This initiative is being pursued by: (1) family transmission and association studies on families identified by a population-based survey for probands with EEG and ERP variants; and (2) a genetic linkage study to map the genes determining the variants. In the genetic epidemiological study, the low voltage alpha (LVA), EEG variant was confirmed to be transmitted in autosomal dominant fashion at least in some families. High and low P300 ERP families have also been identified. Alcoholism and anxiety disorders were more frequent in individuals with the LVA trait, potentially identifying a more homogeneous subgroup of alcoholics with a genetic vulnerability mediated through the anxiety dimension of temperament. Families with the LVA variant, P300 variants, and alcoholism have been collected and families are being analyzed for genetic linkage to dispersed and candidate gene DNA markers.

Project Description:Investigators:

D. Goldman	Chief	LNG, NIAAA
M. Adamson	Senior Clinical Investigator	LNG, NIAAA
L. Akhtar	Chemist	LNG, NIAAA
L. Brown	Clinical Director	LCS, NIAAA
E. Davis	Psychologist	LCS, NIAAA
M. Dean	Staff Scientist	PRI, Frederick, MD
M. Eckardt	Senior Investigator	LCS, NIAAA
R. Ellingson	Assistant Professor	U of Nebraska
M. Enoch	Physician	London, England
C. Harris	Psychologist	LNG, NIAAA
D. Hommer	Section Chief	LCS, NIAAA
J. Long	Special Expert	LNG, NIAAA
S. Michelini	Visiting Fellow	LNG, NIAAA
V. Moore	Social Worker	LCS, NIAAA
J. Rohrbaugh	Assistant Professor	Washington U, St. Louis, MO
K. White	IRTA Fellow	LNG, NIAAA

Objectives:

The main objective is to locate using "reverse genetics" genes responsible for alcoholism and associated behaviors. Genetic vulnerability is an important determinant of alcoholism and other psychopathologies. Application of large numbers of available candidate and dispersed DNA probes as markers for genetic linkage analyses offers the clear possibility of identifying risk genes for these disorders. However, to identify genes for complex, heterogeneous psychiatric diseases, it would be highly advantageous to define genetic psychiatric phenotypes with greater precision and to find phenotypes which correlate more precisely with genotype. One approach to refining the genetic behavioral phenotype is to type neurophysiologic variants relevant to behavior. In alcoholism and other psychopathologies, there is a diminished amplitude of the P300-evoked potential. Variants of the electroencephalogram (EEG), such as the low voltage  $\alpha$  (LVA) variant, have been shown to be transmitted in Mendelian fashion. Evidence indicates that alcoholics have an increased frequency of the LVA variant. We therefore wish to define the prevalence, interrelationships, significance to behavior including alcoholism, genetic transmission, and genetic linkage of variants of the EEG, and variants of event-related potentials (ERPs).

Methods Employed:Psychiatric Assessment

Subjects are clinically assessed in collaboration with Dr. Gerald L. Brown (Section on Family Studies, LCS, NIAAA) and Ms. V. Moore (Section on Family Studies, LCS, NIAAA). Family members receive a comprehensive clinical psychiatric and psychological evaluation, including the SCID psychiatric interview, life history of impulsive behavior, the MAST for alcohol and drug use, a structured family history, and several psychological tests including the MMPI and EPQ (Eysenck Personality Questionnaire). Consensus clinical diagnoses were made using Research Diagnostic Criteria (RDC) by two raters blind to subject identities and incorporating corroborative data from family interviews and from other records.

Ascertainment of EEG Families

For EEG genetic studies, we identified probands with genetic variants of the EEG and studied their families in detail. Subjects were screened for medical conditions or drug use which would interfere with analyses and derived from families with two parents and at least two adult siblings available for analysis. Probands were objectively identified using methods for power spectrum and time

series signal analyses developed by Dr. John Rohrbaugh (Washington U, St. Louis), and others within LCS, NIAAA, and EEGs were also blind-rated impressionistically by an expert in clinical electroencephalography.

#### EEG and ERP Measures

The EEG genetic analyses are performed in collaboration with Drs. Eckardt, Rohrbaugh, and Hommer. The EEG and ERP measures were derived from multiple sites with separate channels for the electrooculogram and heart rate. The resting EEG was obtained for 10 minutes with eyes open, eyes closed, and with the subject hyperventilating. The EEG was subjected to power spectral analysis and was also impressionistically classified using the criteria of Vogel. In the ERP paradigm, subjects experienced two simultaneous trains of stimuli: auditory stimuli consisting of simple tones of varying pitch and visual stimuli derived from the "heads" task developed by Begleiter et al. ERPs were recorded in two situations: in one, the subject identified occasional target visual stimuli whilst ignoring the simultaneous auditory stimuli; conversely, in the second, the rare auditory target was the focus of attention. The paradigm therefore yielded P300s for both auditory and visual stimuli as well as a variety of additional ERP measures related to attention and cognition.

#### For DNA and Protein Genetic Markers

A blood sample was obtained and a lymphoblastoid cell line established. Cells were cultivated to provide sufficient DNA and protein for marker studies and to cryopreserve cells. For genetic linkage, we are testing for nonrandom assortment of marker and EEG phenotype in families, linkage being detected if the two are transmitted as if coupled together or as if in repulsion. Because the likelihood of establishing linkage depends in part on the number of genetic markers typed, a large number of polymorphisms are used. The DNA polymorphisms are primarily variants in the length of cut fragments due to a variable number of copies of short sequences repeated many times in a tandem array (STRs). The STRs are detected by PCR amplification of the DNA with fluorescent primers followed by electrophoresis and fluorescent detection of DNA fragments using an ABI 373A sequencer. On two-dimensional electrophoresis (2DE) protein gels of serum and of lymphoblasts, 25 markers can also be typed and the chromosomal location of the majority of these markers is now known. These polymorphisms, for the most part originally described by us, are variants in isoelectric point.

#### Major Findings:

##### Genetic Linkage in Families with EEG and ERP Variants

This study was designed to determine genetic transmission of hereditary EEG and ERP traits which have been related to alcoholism, to further define their behavioral significance, and ultimately to map genes determining these trait differences. Three phenotypes which we studied were the LVA domain of the resting EEG in which  $\alpha$  (8-12 Hz) activity is absent or greatly diminished, the monomorphic  $\alpha$  (M) resting EEG trait, and the diminished amplitude P300 component of the ERP. Vogel had presented evidence that the LV and M traits had prevalences of approximately 4% and were transmitted in autosomal dominant fashion. Vogel proposed that individuals with the LV phenotype are more likely to become alcoholics. Vogel et al. have also reported psychometric differences between LV and M individuals. However, these results as well as the population and genetic transmission results were unconfirmed. ERPs are at least in part genetically determined. Begleiter et al. and others have found that adult alcoholics and children at risk for alcoholism have a diminished amplitude of the P300 component occurring after stimuli that engage certain types of cognitive activity. The diminished P300 may reflect an impairment in cognitive processing. Little was known about the relationship between P300 and personality traits or to resting EEG traits.

This study differed from previous ones in that it combined population genetic analysis with current methods of EEG spectral analysis to objectify and more accurately define EEG traits, and in that we were able to operationally define



psychiatric diagnosis for all subjects. Our results indicate that the LV variant is a discrete trait occurring in 12.5% of the population. The M variant was found in 9%. As postulated, the LV trait was increased in frequency in alcoholics and individuals with anxiety disorders. Neither trait was age-dependent. The LVA variant was found in only approximately 5% of individuals who were nonalcoholic and without an anxiety disorder. However, LVA was observed in more than 20% of individuals with alcoholism, drug abuse, or anxiety disorders. Of individuals with LVR, 14/17 had an anxiety disorder.

Transmission analysis on a set of eight families indicated that the LV trait was transmitted as an autosomal dominant trait, at least in a subset of the families. The relative likelihood for autosomal dominant transmission versus no genetic transmission was  $>10^{17}$  assuming a genetic penetrance of 0.9 and a population prevalence of the trait of 4%. However, a mixed genetic/environmental mode of transmission could not be ruled out, perhaps due to the limited power of our data set at present.

#### Molecular Probes for Genetic Linkage

The goal of identifying genes whose variants underlie vulnerability towards alcoholism is being pursued in part through hypothesis driven, candidate locus studies (forward genetic approaches) but also by genetic linkage studies in families and well defined populations (reverse genetic approaches). There is considerable interplay between the two approaches; for example, genetic variation at candidate loci involving serotonin and GABA-benzodiazepine function is being sought in families also suitable for genetic linkage using random probes.

For candidate gene analyses, we have identified polymorphisms in the oxytocin receptor, GABAR1, and in a potassium channel gene located on chromosome 20q, where some LVA families have shown genetic linkage. All are detected by polymerase chain reaction of regions of the genes and the GABAR1 variant is a tetranucleotide repeat. The GABAR1 and oxytocin receptor polymorphisms were used to map those genes to their chromosomal locations. As described in other project annual reports, we have also discovered DNA polymorphisms for human tryptophan hydroxylase, ADH5, transketolase, 5-HT<sub>1A</sub>, 5-HT<sub>1D</sub>, 5-HT<sub>1D</sub>, 5-HT<sub>1E</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>2</sub>, and 5-HT<sub>7</sub>, and these markers are available for linkage analyses. Another report also describes short tandem repeat (STR) markers being used in linkage analyses on the EEG families.

#### Significance to Biomedical Research and the Program of the Institute:

Genetically transmitted variants of the EEG are not uncommon. For example, the low voltage a variant is estimated to be present in 5% of the nonanxious, nonalcoholic population and we found a higher frequency of the trait in subjects with alcoholism or with anxiety disorders. The genetically complex psychopathologies which can be addressed using these EEG markers are also relatively common and have a profound impact on population mortality, productivity, and quality of life.

Linkage studies in humans: Familial linkage studies offer a direct method of identifying genetic determinants and markers for vulnerability to alcoholism, bridging the gap between behavioral phenotypes and molecular mechanisms. They also offer a definitive method for testing specific hypotheses regarding the effects of variants at candidate genetic loci including neurotransmitter biosynthetic, receptor, and alcohol metabolic loci.

EEG: The clinical and electrophysiological phenotyping of families has allowed us to identify a subgroup of alcoholics with anxiety disorders and an EEG phenotype which is often transmitted in autosomal dominant Mendelian fashion. The cell lines we have prepared from the members of these families offer a permanent source of DNA for the linkage analyses which can help us to identify a gene for this trait.

Proposed Course:

We will continue to type candidate and random dispersed genetic markers in families with the LVA variant of the EEG in order to identify a gene or genes determining this trait.

Collection of families with EEG and P300 ERP variants which have been related to alcoholism will continue to provide additional material for linkage analyses and for studies on genetic transmission and quantitative gene effects on EEG parameters.

We will further assess the interrelationship between different variants of the EEG and ERP and the relationships of these variants to behavioral traits including alcoholism.

Publications:

Durcan MJ, Goldman D. Genomic imprinting: Implications for behavioral genetics, J Behavioral Genetics 1993;23:137-43.

Michellini S, Urbanek M, Goldman D. Polymorphism and genetic mapping of the human oxytocin receptor gene on chromosome 3, Neuropsychiatric Genetics, in press.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00281-05 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Genetic Studies on Alcoholism in American Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. Goldman Chief LNG, NIAAA

Others: L. Akhtar Chemist LNG, NIAAA  
G. Brown Clinical Director LCS, NIAAA  
M. Linnoila Scientific Director NIAAA  
J. Long Specific Expert LNG, NIAAA  
R. Robin Senior Staff Fellow LNG, NIAAA  
J. Washa Social Science Analyst LNG, NIAAA

COOPERATING UNITS (if any)

PRI, Frederick, MD (M. Dean); Ctr Human Behav Std (B. Albaugh); PECR, NIDDK (P. Bennett, W. Knowler); Ramsey Med Ctr (J. Jaranson); Nat Ctr Amer Indian AK Nat Ment Hlth Res (S. Manson, J. Shore, J. Beals); UCLA (B. Caldwell); U AZ (A. Chang)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Human Neurogenetics

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

4.0

PROFESSIONAL:

2.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To identify alcoholism risk genes, we are collecting and testing for linkage families from three different American Indian populations, which are relatively homogeneous and in which alcoholism is highly prevalent. This study also addresses the genetic epidemiology and psychiatry comorbidity of alcoholism in American Indians using structured psychiatric diagnostic interviews of subjects in large families. Studies of Cheyenne and Pima Indians are currently in progress. Two hundred Cheyenne Indian and 500 Pima Indian subjects from large families have been clinically evaluated and their cell lines immortalized with a target family size of 600 from each community. At Jemez Pueblo, data and cell lines have been collected for 100 of 200 subjects. A large panel of random polymorphic probes has been typed in Cheyenne alcoholics and controls and genetic linkage analysis is in progress for this group. An analysis of psychiatric comorbidity in Cheyenne and Pima Indian alcoholics and nonalcoholics has been completed.

Project Description:Investigators:

D. Goldman	Chief	LNG, NIAAA
L. Akhtar	Chemist	LNG, NIAAA
B. Albaugh	Director of Research	CHBS
		Weatherford, OK
J. Beals	Associate Director	NCAINMHR
P. Bennett	Chief	PECR, NIDDK
G. Brown	Clinical Director	LCS, NIAAA
B. Caldwell	Clinical Professor	UCLA
A. Chang	Faculty	U of Arizona
M. Dean	Staff Scientist	PRI, Frederick, MD
J. Jaranson	Clinical Director	Ramsey Medical Center
		St. Paul, MN
W. Knowler	Section Chief	PECR, NIDDK
M. Linnoila	Scientific Director	NIAAA
J. Long	Special Expert	LNG, NIAAA
S. Manson	Director	NCAINMHR
R. Robin	Senior Staff Fellow	LNG, NIAAA
J. Shore		NCAINMHR
J. Washa	Social Science Analyst	LNG, NIAAA

Objectives:

This is a study on alcoholism in families derived from three American Indian populations. There are three primary objectives: (1) to better characterize the clinical phenotype of alcoholism, including psychiatric comorbidity; (2) to ascertain the extent to which alcoholism in these Indian groups is familial; and (3) in large families, to locate using dispersed and candidate genetic markers the genes responsible for alcoholism vulnerability. The candidate loci include genes connected to serotonin function and ethanol metabolism as well as markers for alcoholism defined in other populations.

Methods Employed:

All subjects are psychiatrically interviewed by a psychologist (R. Robin) or psychiatrically trained social worker (B. Albaugh). Clinical assessment is in collaboration with J. Jaranson (Ramsey Medical Center) and G. Brown (LCS, NIAAA). Family members receive a comprehensive clinical, sociocultural, and psychological evaluation, including the SADS-L structured psychiatric interview, the MAST for alcohol and drug use, a structured family history, and several psychological tests including the MMPI. A consensus clinical diagnosis is made by two raters blind to subject identities and incorporating data from corroborative family interviews, the MAST and other records.

For DNA and protein genetic markers, a blood sample is obtained and a lymphoblastoid cell line is established. Cells are cultivated to provide sufficient DNA and protein for marker studies and to cryopreserve cells.

For genetic linkage, we test for nonrandom assortment of marker and phenotype in families, linkage existing if the two are transmitted as if coupled together or as if in repulsion. (See project Z01 AA 00016-02 LNG: "Gene Mapping and Linkage Studies with Short Tandem Repeat (STR) Markers"). Population associations are also sought.

Major Findings:DRD2 Dopamine Receptor Genotype

The frequency of the DRD2 dopamine receptor Tag1/A1 marker, reported to be associated with alcoholism, was four times higher in Cheyenne Indians as compared

to Caucasians and it was also several times as abundant in Jemez Pueblo Indians. Despite the high frequency of the marker in Cheyenne Indians, DRD2 was not associated with alcoholism in this population. However, linkage disequilibrium between the TaqI polymorphism located >10 kb downstream from DRD2 to a marker in the immediate 3' region of DRD2 was significantly higher in Cheyenne Indians as compared to two Caucasian populations. These results indicate the increased power of performing linkage and association studies in genetically more homogeneous populations (and large families) in which ethnic stratification of patients and controls will not be a problem and in which marker associations may often be stronger.

#### Psychiatric Comorbidity in American Indian Alcoholics

A population of 159 Cheyenne Indians was assessed for comorbidity of psychiatric disorders using the SADS-L psychiatric interview. This sample, consisting of community volunteers and their relatives, included 60 males, of whom 68% were alcoholic and 99 females, of whom 38% were alcoholic. Although this was not an epidemiologic survey, the lifetime prevalence of alcoholism found in the present study sample (49%) is consistent with the high reported prevalence of alcoholism among the Cheyenne. Furthermore, these community-identified alcoholics can be evaluated for prevalence of other problems.

No significant differences were found when results were compared between related (first degree relatives) and an unrelated subsample of subjects, so we focus on the level of psychiatric comorbidity in the whole sample. Most striking were the high rates of major psychiatric disorder (56%) and multiple psychiatric disorders (20%) among these community alcoholics. Conversely, only 18% of nonalcoholics had a major lifetime psychiatric disorder and the vast majority of these were women with depression. Only 4% of nonalcoholics had multiple psychiatric disorders. Alcoholics were more than five times as likely to develop a psychiatric disorder than nonalcoholics (odds ratio analysis). They also had more than twice the risk of developing depression and ten times the risk of drug abuse. Analysis of prevalence rates of depression for males and females and alcoholics and nonalcoholics suggests that the etiology and strength of association between alcoholism and depression may differ between males and females. The Cheyenne sample had a substantial number of subjects with drug abuse (14%) compared to most American Indian and non-Indian populations. The finding that 27% of Cheyenne alcoholics have abused drugs and that 20/22 (91%) of the drug abusers were alcoholics suggests a strong association between the two disorders. Female alcoholics were as likely to have drug abuse (29%) as were male alcoholics (22%). All Cheyenne subjects in this sample with antisocial personality (N=7) were also alcoholic and all were male, indicating that there may be a subgroup of Cheyenne alcoholics with antisocial personality and male limited expression, as postulated for other populations. Overall, there appears to be a clustering of depression, drug abuse, and antisocial personality with alcoholism. It is clear that this clustering is not entirely due to psychopathology occurring in the context of drinking.

The literature contains frequent reference to American Indian drinking styles characterized by binge or episodic drinking (defined by Research Diagnostic Criteria as  $\geq 3$  consecutive days of heavy drinking on  $\geq 3$  separate occasions). Cheyenne subjects were assessed according to four patterns of drinking: (1) binge drinking only; (2) binge, and heavy, consistent drinking (intoxicated each week  $\geq 4$  consecutive weeks); (3) heavy, consistent drinking and no binge; and (4) no heavy, consistent drinking and no binge. Preliminary results indicate that approximately 40% of the Cheyenne subjects engaged in binge drinking. All of the binge drinkers also had periods of more consistent, heavy drinking; there were no "pure" binge drinkers. Only 10% of the subjects had patterns of consistent, heavy drinking without binge episodes. Thirty-eight percent (38%) of the subjects had no evidence of either binge or consistent, heavy drinking patterns. A comparison of Cheyenne subjects who met criteria for these different patterns of drinking provided some interesting results. Binge/heavy drinkers were significantly more likely to be divorced or separated, to have few close social



or familial relationships, to develop physical symptoms of alcohol abuse and to experience withdrawal symptoms, to have conflicts with the police and to act aggressively, to develop alcoholism at an early age, and to engage in heavy drinking over a longer period of time as compared to subjects who drank heavy and consistently but who did not binge and as compared to subjects who did not drink heavily. These findings suggest that the associated phenomenology and, perhaps, the etiology of alcoholism, may be different for individuals with substantially different drinking patterns. Alcoholism is most likely a complex behavior having different etiologies.

#### Significance to Biomedical Research and the Program of the Institute:

Familial linkage studies offer a direct method for bridging the gap between fundamental molecular mechanisms and behavioral phenotypes related to the vulnerability to alcoholism. Genes for alcoholism will be difficult to map in Caucasian families because alcoholism is clinically (and probably genetically) heterogeneous, environmentally influenced, highly prevalent, and marked by assortative mating. The greater environmental and genetic homogeneity of these Indian communities greatly increases the likelihood of mapping genetic loci or identifying factors predisposing individuals to alcoholism. The families and populations detected here will also provide useful data sets for testing the generalizability of genetic linkages found in other populations.

For many American Indian communities, alcohol has a tragic impact. Alcoholism accounts for more than 50% of deaths in the three communities in our study, and alcoholism also degrades the quality of family and social relationships at multiple levels. Careful, structured, and in depth evaluations will enable us to accurately compare the clinical phenomenology of alcoholism in these American Indian populations with each other and to Caucasians. By defining age of onset and severity, by accurately defining psychiatric comorbidity, and by following the transmission of pathologies in families, we can gain a better understanding as to the causes of alcoholism and derive more effective assessment, treatment, and prevention approaches.

#### Proposed Course:

An additional 200 Pima Indian clinical interviews from the same large family will be blind-rated and diagnoses and DNA available early in 1994, for a total of 600 on whom full data will be available by the end of 1995. The Cheyenne Indian family is being expanded so that completed blind-ratings for psychiatric diagnoses and DNA should be available on more than 400 subjects by the end of 1995, enabling us to conduct analyses for genetic linkage and clinical genetic associations to alcoholism. During this year, clinical data collection will be directed towards comparison of population samples from tribes with high and low rates of alcoholism. These population samples will be compared for genetic and sociobiologic factors which may have determined their differential vulnerabilities. The populations will also be contrasted for alcoholism-related problems, including family violence.

#### Publications:

Chester B, Robin RW, Koss M, Lopez J, Goldman D. Grandmother dishonored: Violence against women by partners in American Indian communities. In: Urquiza A, Wyatt G, Root M, eds. Violence against women of color. Violence and victims (special issue): Sacramento, California, in press.

Dean M, Stevens JP, Winkler C, Lomb DA, Ramsburg JJ, Boaze R, Steward C, Charbonneau L, Goldman D, Albaugh BJ, Goedard JJ, Beasley P, Hwang L-Y, Buckbinder S, Kaslow RA, O'brien IB, Gomperts E, Donfield S, Johnson PA, Eichelberger M, O'Brien SJ. Polymorphic admixture typing in human ethnic populations, *Am J Hum Genet*, in press.



Goldman D, Brown GL, Albaugh B, Robin R, Goodson S, Trunzo M, Akhtar L, Wynne DK, Lucas-Derse S, Bolos A, Tokola R, Virkkunen M, Dean M. D2 dopamine receptor genotype, linkage, disequilibrium and dopamine function in Finnish, American Indian and U.S. Caucasian alcoholics and the population association approach in psychogenetics. In: Gershon E, Cloninger C, eds. Genetic approaches to mental disorders. Washington, DC: American Psychiatric Press, 1994;327-44.

Robin RW, Chester B, Goldman D. Cumulative trauma in American Indian communities. In: Marsella A, ed. Ethno-cultural issues in post-traumatic stress disorder. American Psychiatric Association Press, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00282-05 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Genetic Studies on the Dopamine D2 Receptor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D. Goldman Chief LNG, NIAAA

Others: M. Linnoila Scientific Director NIAAA

R. Robin Senior Staff Fellow LNG, NIAAA

COOPERATING UNITS (if any)

Program Resources Inc., Frederick, MD (M. Dean); U of Helsinki (R. Tokola, M. Virkkunen); Washington University (A. Bolos); Ctr for Behav Res (B. Albaugh)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Human Neurogenetics

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

Goldman D. Commentary on genetic heterogeneity and genetic etiologies for alcoholism and substance abuse, Annual Review of Drug Abuse and Addictions 1992;217-21.

Goldman D, Dean M, Brown GL, Bolos AM, Tokola R, Virkkunen M, Linnoila M. D2 dopamine receptor genotype and cerebrospinal fluid homovanillic acid, 5-hydroxyindoleacetic acid and 3-methyl-4-hydroxyphenylglycol in Finnish and American alcoholics, Acta Psychiatr Scand 1992;86:351-7.

Goldman D, Brown GL, Albaugh B, Robin R, Goodson S, Trunzo M, Akhtar I, Wynne DK, Lucas-Derse S, Bolos AM, Tokola R, Virkkunen N, Linnoila M, Dean M. D2 dopamine receptor genotype, linkage disequilibrium, and dopamine function in Finnish, American Indian, and U.S. Caucasian alcoholics and the population association approach in psychogenetics. In: Gershon E, Cloninger C, eds. Genetic approaches to mental disorders. Washington, DC: American Psychiatric Press, 1994;327-44.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00290-04 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Genetic Studies of Disturbed Serotonin Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. Goldman	Chief	LNG, NIAAA
Others:	M. Adamson	Senior Clinical Investigator	LNG, NIAAA
	M. Koulou	Visiting Scientist	LNG, NIAAA
	J. Lappalainen	Visiting Fellow	LNG, NIAAA
	M. Linnoila	Scientific Director	NIAAA
	J. Long	Special Expert	LNG, NIAAA
	D. Nielsen	Senior Staff Fellow	LNG, NIAAA
	N. Ozaki	Visiting Fellow	LNG, NIAAA

COOPERATING UNITS (if any)

U Helsinki (M. Virkkunen, M. Eggert); Program Resources Inc., Frederick, MD (M. Dean); Washington U (M. Pranzatelli); Inst. Behav. Gen. (T. Johnson); U Pittsburgh (W. Kaye); Lab Clin Stds, NIMH (D. Murphy); Clin Psychobiol Br, NIMH (N. Rosenthal)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Human Neurogenetics

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOXES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Studies on individuals and animals with genetic defects in serotonin function can shed light on the role of this neurotransmitter in behavior and on the role of milder functional variants in serotonin genes in predisposing individuals to psychopathologies and to alcoholism. We are identifying probands for family studies by measuring the serotonin metabolite 5-HIAA in cerebrospinal fluid and by identifying individuals with amino acid substitutions in genes involved with serotonin function. Two 5-HT1A variants are rare amino acid substitutions (22Gly--Ser and 28Val--Ile), one conservative and one nonconservative. The 5-HT2C variant is a common (allele frequency=0.18) nonconservative substitution (Cys--Ser). For association and direct gene analysis, we have collected more than 40 cell lines from each of the following populations: anorexia nervosa (collaboratively with W. Kaye), obsessive compulsive disorder (D. Murphy), low CSF 5-HIAA with Type II alcoholism (M. Linnoila, M. Virkkunen, M. Eggert), and seasonal affective disorder (N. Rosenthal, N. Ozaki). The detected polymorphisms are converted to PCR RFLPs or allele-specific amplification markers for ease of analysis. Using the CEPH reference pedigrees and the polymorphisms at these genes, each gene is genetically mapped to its chromosomal location. For direct gene analysis, we mainly use single-strand conformational polymorphism analysis and direct sequencing. A TPH polymorphism was found to be associated with low CSF 5-HIAA and suicidality in impulsive alcoholic Finns. For serotonin receptors, coding sequence polymorphisms of 5-HT1A, 5-HT1D $\alpha$ , 5-HT1D $\beta$ , 5-HT2C, 5-HT2A, 5-HT1E, and 5-HT7 were identified.



Project Description:Investigators:

D. Goldman	Chief	LNG, NIAAA
M. Adamson	Senior Clinical Investigator	LNG, NIAAA
M. Dean	Staff Scientist	PRI, Frederick MD
M. Eggert	Staff Physician	U Helsinki, Finland
T. Johnson		Institute for Behavioral Genetics U of Pittsburgh
W. Kaye		LNG, NIAAA
M. Koulu	Visiting Scientist	LNG, NIAAA
J. Lappalainen	Visiting Fellow	LNG, NIAAA
M. Linnoila	Scientific Director	NIAAA
J. Long	Special Expert	LNG, NIAAA
D. Murphy	Chief	LCS, NIMH
D. Nielsen	Senior Staff Fellow	LNG, NIAAA
N. Ozaki	Visiting Fellow	LNG, NIAAA
U. Pesonen	Visiting Fellow	LNG, NIAAA
M. Pranzatelli		George Washington U
N. Rosenthal	Section Chief	CPB, NIMH
M. Virkkunen	Senior Lecturer	U Helsinki, Finland
F. Weight	Chief	LMCN, NIAAA

Objectives:

The role serotonin plays in developing and controlling behavior is incompletely defined, although the importance of this neurotransmitter in alcoholism and in other psychopathologies is clear. In this study, we are attempting to gain insights into normal serotonin gene function and the effects of gene variants by identifying serotonin gene polymorphisms and studying individuals with these variants. Although serotonin modulates a number of important behaviors including sleep, impulsivity, appetite, and body temperature as well as ethanol intake, no human or animal behavioral mutants in serotonin function had previously been identified. It is therefore logical that abnormalities of serotonin function may cause unknown effects in adult tissues and during development. In this study, we are: (1) identifying patients with biochemical genetic deficits in serotonin function and establishing lymphoblastoid cell lines on these patients and members of their families; (2) evaluating serotonin candidate genes for genetic variation using SSCP (single-strand conformational polymorphism) analysis and direct sequencing; and (3) correlating genotype with phenotype.

Methods Employed:Selection of Probands

Children and adults with evidence of a serotonergic genetic disorder either by family history or the presence of significant dysmorphology are screened. Candidate serotonergic genetic disorders being screened include: (1) anorexia nervosa, (2) hereditary myoclonus, (3) seasonal affective disorder, (4) obsessive-compulsive disorder, (5) alcoholism associated with impulsivity and low CSF 5-HIAA, and (6) other serotonin-associated behavioral disorders. Medical, neuropsychiatric, and genetic evaluation is performed on these subjects. Monoamine and monoamine metabolite levels are obtained from cerebrospinal fluid in some subjects. CSF measures of monoamines, monoamine metabolites, and neuropeptides are obtained after a standard three day low monoamine diet and lumbar puncture under carefully controlled conditions.

For DNA and protein genetic marker studies, venipuncture is performed and a lymphoblastoid cell line is established. For direct analysis of genes involved in serotonin function, DNA is amplified by PCR using primers that are fluorescently or radioisotopically tagged. DNA is then either direct sequenced using an automated sequencer or is evaluated for sequence variation using the

single-strand conformational polymorphism (SSCP) method in which the single-stranded DNA is electrophoresed under nondenaturing conditions.

The following genes are being analyzed: 5-HT<sub>1A</sub>, 5-HT<sub>1D $\alpha$</sub> , 5-HT<sub>1D $\beta$</sub> , 5-HT<sub>1E</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>3</sub>, 5-HT<sub>7</sub>, and TPH.

#### Major Findings:

Although behavior is ultimately controlled by numerous genes and other causative factors, better clinical subtyping and the study of behaviorally extreme individuals can result in the identification of discrete genetic entities. Our approach to find genetic phenotypes due to serotonin dysfunction has been to study individuals with behavioral traits and neurochemical abnormalities related to serotonin function. A large collection of cell lines and DNA samples has been assembled which includes large numbers of alcoholics with low and high levels of CSF 5-HIAA. These subjects are obviously very useful for mutation screening studies. For ease of analysis, PCR RFLP and allele-specific amplification (ASA) assays are developed for the DNA variants. To establish the location of these genes on the human linkage map, CEPH reference pedigrees are typed for the polymorphisms found at each locus and linkage is calculated using two locus and multiple locus methods versus the marker data previously entered in the reference database. Through collaborations and for the purpose of direct mutation scanning, we have also collected DNA samples and cell lines from patients with eating disorders, hereditary myoclonus, obsessive-compulsive disease, and seasonal affective disorder.

Polymorphisms of the TPH gene in humans, mice, and rhesus macaques described by D. Nielsen in the lab are being used in a number of genetic association and linkage studies. The human TPH variant was shown to be due to a point mutation within an intron. This TPH variant was used to map TPH to its location on chromosome 11p, near hemoglobin B. A collection of impulsive and nonimpulsive alcoholic, violent offenders and healthy volunteers was analyzed by D. Nielsen in collaboration with M. Virkkunen and R. Tokola (U of Helsinki). A significant association was found between TPH genotype and both suicidality and CSF 5-HIAA levels. DNA samples from impulsive individuals are currently being analyzed for functional variants of TPH by direct sequencing and by SSCP analysis of coding sequence.

We have also detected novel polymorphisms within the coding sequences of eight serotonin receptor genes, 5-HT<sub>1A</sub>, 5-HT<sub>1D $\alpha$</sub> , 5-HT<sub>1D $\beta$</sub> , 5-HT<sub>1E</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>3</sub>, and 5-HT<sub>7</sub>. Using SSCP analysis, J. Lappalainen discovered a common polymorphism (rare allele=0.40) within the coding sequence of 5-HT<sub>1D $\beta$</sub> . It is a synonymous mutation but has already been useful in precision linkage mapping of this locus, which is found on chromosome 6. Recently, J. Lappalainen found a 5-HT<sub>1E</sub> variant and demonstrated that this gene maps near to the same location. This may provide the first example of physical clustering in the 5-HT receptor gene family. A variant of 5-HT<sub>1D $\alpha$</sub>  was discovered by N. Ozaki and J. Lappalainen. Sequencing of the 5-HT<sub>1D $\alpha$</sub>  variant has revealed it also to be nonfunctional and this gene was mapped on chromosome 1. Rapid assays have been developed for both 5-HT<sub>1D</sub> variants by N. Ozaki and J. Lappalainen. These serotonin receptor variants are being used in association and linkage studies while we continue to search for functional variants at these loci. Incompletely characterized variants at 5-HT<sub>2A</sub> and 5-HT<sub>7</sub> have been found by U. Pesonen and M. Koulu.

Two 5-HT<sub>1A</sub> coding sequence variants were detected by B. Nakhai. Both encode amino acid substitutions located in the N-terminal region of the protein and in the extracellular domain. They are 22Gly-->Ser and 28Val-->Ile. Dr. Nakhai has inserted these mutations into an expression construct and is comparing them to the wild-type allele for functional differences in ligand binding and signal transduction.

A common (allele frequency=0.18) 5-HT<sub>x</sub> variant was found by J. Lappalainen. This variant encodes a Cys-->Ser nonconservative amino acid substitution. This gene was mapped to its location on the X chromosome. Collaboratively with F. Weight and L. Chang, this variant has been expressed in *Xenopus* oocytes so that it can be compared to the wild-type sequence for functional differences.

In the area of animal studies, collaboratively with D. Higley (LCS, NIAAA), we are performing genetic association studies. SSCP variants we have identified at the TPH and 5-HT<sub>1A</sub> (serotonin receptor) loci. With T. Johnson (Institute for Behavioral Genetics, Boulder, CO), we have performed a linkage study using the LS/SS recombinant inbred lines. This study revealed that TPH was a quantitative trait locus (QTL) accounting for 6% of the variance in sleep time following the injection of ethanol. This is the largest QTL thus far discovered for this trait.

#### Significance to Biomedical Research and the Program of the Institute:

Diminished turnover of serotonin is associated with impulsive behavior and appears to be related to alcoholism risk. Because of the diverse functions of serotonin in regulating sleep, appetite, mood and body temperature, it is highly likely that genetic variants of serotonin genes may be important in a variety of disorders such as eating disorders, obsessive-compulsive disease, and antisocial personality. The association we have found between TPH genotype and suicidality and 5-HIAA levels in the impulsive Finns may indicate that a functional variant of TPH is relatively common in this relatively homogeneous and extreme subgroup of alcoholics.

The two rare amino acid substitutions found at 5-HT<sub>1A</sub> and the abundant nonconservative amino acid substitution found at 5-HT<sub>x</sub> are the first variants of serotonin receptors described which could directly affect the behavioral phenotype.

#### Proposed Course:

We will continue to directly analyze genes involved in serotonin function in DNAs from alcoholics and other individuals who appear to be genetically abnormal in serotonin function. We will seek to correlate the clinical phenotype to the genotype and in appropriate families, we will perform genetic transmission and linkage analysis. To assess functionality of amino acid substitutions, the variants will be assayed in appropriate *in vitro* expression systems.

#### Publications:

Bolos AM, Goldman D, Dean M. An Alu repeat polymorphism at the 5-hydroxytryptamine 1A receptor (HTR1A) gene, *Psychiatric Genetics* 1993;3:235-40.

Ellison J, Dean M, Goldman D. Efficacy of fluorescence-based PCR-SSCP for detection of point mutations, *Biotechniques* 1993;684-91.

Ellison J, Squires G, Goldman D. Detection of mutations and polymorphisms using fluorescence-based dideoxy fingerprinting (F-ddF), *Biotechniques*, in press.

Goldman D. The search for alleles contributing to self-destructive and aggressive behaviors. In: Stoff D, Cairns R, eds. *The neurobiology of clinical aggression*, in press.

Higley JD, Thompson WT, Champoux M, Goldman D, Hasert MF, Kraemer GW, Scanlan JM, Suomi SJ, Linnoila M. Paternal and maternal genetic contributions to CSF monoamine metabolites in rhesus monkeys (*Macaca mulatta*), *Arch Gen Psychiatry* 1993;50:615-23.

Hulihan-Giblin BA, Park Y, Aulakh CS, Goldman D. Regional analysis of 5-HT<sub>1A</sub> and 5-HT<sub>2</sub> brain receptors in Fawn-Hooded, Sprague-Dawley and Wistar male rats, Eur J Pharmacol, in press.

Hulihan-Giblin BA, Park Y, Pivorun EB, Goldman D. Regional analysis of 5-HT<sub>1A</sub> receptors in two species of *Peromyscus*, Pharmacol Biochem Behav 1993;45:143-5.

Hulihan-Giblin BA, Pivorun EB, Goldman D. Diurnal variation of 5-HT<sub>1A</sub> and 5-HT<sub>2</sub> receptor binding in euthermic and torpor prone deer mice, *Peromyscus maniculatus*, Pharmacol Biochem Behav 1993;45:785-9.

Lappalainen J, Dean M, Charbonneau L, Virkkunen M, Linnoila M, Goldman D. The mapping of the 5-HT<sub>1D $\beta$</sub>  autoreceptor gene on chromosome 6 and direct analysis for sequence variants, Neuropsychiatric Genetics, in press.

Ozaki N, Lappalainen J, Dean M, Virkkunen M, Linnoila M, Goldman D. Mapping of the serotonin 5-HT<sub>1D $\beta$</sub>  autoreceptor gene on chromosome 1 using a coding sequence polymorphism, Psychiatric Genetics, in press.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 AA 00016-02 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Gene Mapping and Linkage Studies with Short Tandem Repeat (STR) Markers**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Long Special Expert LNG, NIAAA

Others: D. Goldman Chief LNG, NIAAA

E. Moore Computer Specialist LNG, NIAAA

A. Novoradovsky Visiting Associate LNG, NIAAA

M. Urbanek IRTA Fellow LNG, NIAAA

COOPERATING UNITS (if any)

University of New Mexico (F. Romero)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Human Neurogenetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

3.5

PROFESSIONAL:

3.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are searching for genetic linkage between short tandem repeat (STR) markers and genes that contribute to the predisposition to alcoholism and related behaviors. This involves development of a battery of 350 highly polymorphic marker loci that span the human genome at approximately 10-15 centimorgan intervals. We are using fluorescent dye technology and constructing panels of 10-20 loci that can be run simultaneously in one gel lane. This will facilitate fast and accurate data collection. Computer programs are being written to interface between the gel analysis machinery and statistical databases. These programs save time and reduce the potential for data entry and retrieval errors. In addition, we are developing methods for PCR-STR optimization and for precise STR allele determinations. As more genotypes are collected in the next year, we will begin linkage analyses using sequential likelihood and affected relative pair methods. The informativeness of STR markers bestows new and heretofore unattainable power for establishing, and excluding, genetic linkage between definable chromosome segments and genetic vulnerability to disease.

Project Description:Investigators:

J. Long	Special Expert	LNG, NIAAA
D. Goldman	Chief	LNG, NIAAA
E. Moore	Computer Specialist	LNG, NIAAA
A. Novoradovsky	Visiting Associate	LNG, NIAAA
F. Romero		U of New Mexico
M. Urbanek	IRTA Fellow	LNG, NIAAA

Objectives:

We are searching for short tandem repeat (STR) DNA markers linked to genes conferring vulnerability to alcoholism and related behavioral phenotypes. STR polymorphisms have many alleles per locus and they are abundantly distributed throughout the genome. Because of these properties, they are the most promising class of genetic polymorphism to yield successful linkage results. Our first major goal is to develop for linkage analysis a panel of STR marker genes that are highly polymorphic and evenly spaced at approximately 10 centimorgan (cM) intervals along the human chromosomes. In pursuit of this goal, we are: (i) developing multiplex procedures that enable sets of marker loci to be simultaneously run on a single gel lane using an Applied Biosystems 373a automated DNA sequencer, (ii) automating data collection by interfacing the Applied Biosystems Genescan 672 and Genotyper software kits with the Microsoft Excel spread sheet package and custom written Pascal programs, and (iii) developing a database management system that facilitates easy storage, retrieval, and transfer to analytical software applications. Our second major goal is to analyze large pedigrees for linkage between STR loci and alcoholism and related behaviors. For this purpose, we are making computer links between our genetic marker databases and family structure databases so that meaningful data subsets can be extracted and fed into pedigree drawing and genetic linkage analysis software.

Methods Employed:Marker Loci

We are collaborating with Applied Biosystems Division of Perkin Elmer (ABD) to develop fluorescent dye-labeled polymerase chain reaction (PCR) primers for 386 STR loci. These loci have been selected from the Genethon set. Their heterozygosities are generally above 75% and the genetic distance between adjacent markers is less than 10 cM on the average. In addition to the ABD collaboration, we have developed primers for about 45 loci. These loci have somewhat lower heterozygosities, but they usefully increase the linkage map density. Information about the marker loci is stored in a database which assigns a 15 field record to each locus. The fields contain summary information such as chromosomal locations, upper and lower primer sequences, annealing temperatures, repeat unit sizes, numbers and sizes of alleles, etc.

Molecular Biology

Locus-specific fragments are amplified from genomic DNA using the PCR with dye-labeled primers. Alleles are identified by size sorting during electrophoretic separation in denaturing polyacrylamide gels using an Applied Biosystems 373a automated DNA sequencer which employs fluorescent dye detection and allows analysis of several loci and internal size standards in a single lane. We have interfaced the Applied Biosystems Genescan 672 software with Microsoft Excel so that data can be transferred directly into marker data files that link the battery of molecular typings with individuals. We are also using the ABD genotyper software package for automated and assisted genotype calling.

Databases

The molecular genetic data records have a subject identification field that allows computer links with our family structure files. Programs have been written to extract pedigrees and subsets of pedigrees from the family structure files and feed them into pedigree drawing (PedDraw) and genetic analysis (S.A.G.E. and LINKAGE) computer programs.

Statistical Methods

A variety of genetic linkage analyses are being performed using these data. Two point and multipoint linkage analyses are carried out using the parametric likelihood methods contained in Lathrop's LINKAGE program and the Statistical Analysis in Genetic Epidemiology (SAGE) package. These programs allow age-of-onset and penetrance parameters to be estimated using the pedigree data. Robust sibling pair linkage methods are also being applied because of uncertain transmission modes for alcoholism and psychopathologies. In addition to finding linkages to disease predisposing loci, we are intent on excluding regions without linked genes.

Major Findings:Analysis of Measurement Error

In order to determine whether or not STR alleles can be scored with sufficient accuracy so that data from different gels are directly comparable, a replication study was performed. Six members of one nuclear family were selected, DNA from each person was amplified by two separate PCR reactions for 16 STR loci. Two aliquots from each PCR were run on each of two gels. DNA fragment size alleles were determined in the parents and confirmed in the offspring. The percentages of miscalled genotypes and alleles were calculated by direct counting. Analysis of variance (ANOVA) was performed to determine whether fragment size measurements for an allele varied systematically with respect to the gel, PCR, or subject (i.e., genotype). Forty alleles were distributed among the 16 loci. The error rate for allele determinations was  $0.9\% \pm 0.3\%$ , and for genotype determinations it was  $1.2\% \pm 0.4\%$ . A significant effect of electrophoretic gel on size determination was observed for eight out of the 40 alleles. However, these effects were generally small ( $<0.25$  base pairs) and not so great as to affect the accuracy of allelic determinations from gel to gel. The PCR reaction had a significant effect for only three of the 40 alleles. Once again, these effects are insufficient to affect intergel allelic determinations. The subject that the allele was drawn from did not affect the size determination for any of the 40 alleles.

STR Allele Population Diversity

Five STR makers panels consisting of a total of 45 loci have been used to analyze a variety of populations including 125 Cheyenne Indians which were typed with 30 markers, 200 Koreans typed with 20 markers, 180 Pima Indians typed with 15 markers, 120 Pueblo Indians typed with 10 markers, 40 Navajo typed with 15 markers, and 40 Bethesda Caucasians typed with 15 markers. The analysis of these typings showed that: (1) most of the alleles of a specific marker were present in several populations although the presence of some alleles appears to be population-specific and the frequency of alleles can vary dramatically between populations, and that (2) the observed heterozygosity of the markers corresponded well with the published values. Although the level heterozygosity for some of the markers was somewhat reduced in our population isolates, the heterozygosity values were still predominantly above 0.60 making the markers useful for linkage studies in our populations.

Genetic Map Distance and Linkage Disequilibrium

In order to test the generality of linkage disequilibrium mapping, we examined patterns of linkage disequilibrium between pairs of STR loci within a known genetic map. Two populations were analyzed. The first population, Navajo Indians (N=45), is an isolate that experienced a severe bottleneck in the 1860s. The second population, Maryland Caucasians (N=45), is cosmopolitan. We expected



the Navajo sample to display more linkage disequilibrium than the Caucasian sample, and possibly that the Navajo disequilibrium pattern would reflect the genetic map. Fifteen highly polymorphic dinucleotide repeat loci spanning chromosome 20 at 5-10 cM intervals were typed using an ABI 373A DNA sequencer (Foster City, CA) following PCR amplification with fluorescent dye-labeled primers. Linkage disequilibrium coefficients were estimated between all pairs of alleles at different loci using maximum likelihood. The net disequilibrium between pairs of loci was measured as a weighted average of individual coefficients. The genetic isolate structure of Navajo Indians is confirmed by the DNA typings. Heterozygosity is lower than in the Caucasians, and fewer different alleles are observed. However, a relationship between genetic map distance and linkage disequilibrium could be discerned in neither the Navajo nor the Maryland samples. Slightly more linkage disequilibrium was observed in the Navajos, but both data sets were characterized by very low disequilibrium levels. We tentatively conclude that linkage disequilibrium mapping with dinucleotide repeats will only be useful with close linkage between markers and diseases, even in very isolated populations.

#### Significance to Biomedical Research and the Program of the Institute:

Family twin and adoption studies have confirmed a genetic contribution to the transmission of alcoholism vulnerability. The next step is to identify traits, or gene products, that either directly contribute to the pathophysiology of the disease, or are closely linked to genes that do. With the exception of the protective effect of the ADH2 and ALDH polymorphisms, little is known about such genes. Transmission and gene mapping studies have been difficult because alcoholism is a common, complex and genetically heterogeneous disease. Utilization of our battery of highly polymorphic and closely linked markers will add enormous power to genetic linkage analyses.

#### Proposed Course:

Our objectives in the next year are to: (1) complete 350,000 typings/year at 7,000 typings/week, (2) test five more panels for our collaboration with ABD, (3) begin typing of the complete set ABD marker panel as it becomes available, (4) develop multiplex PCR amplification, so that reagent costs and protocol times are reduced, (5) type Cheyenne Indian, Pima Indian, and Finnish alcoholics, and their families for the DNA markers, and (6) perform linkage analyses on these data using likelihood and affected relative pair methods.

#### Publications:

Michellini S, Urbanek M, Dean M, and Goldman D. Polymorphism and genetic mapping of the human oxytocin receptor gene on chromosome 3, *Neuropsychiatric Genetics*, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00017-02 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Population Genetics of Native American Tribes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Long Special Expert LNG, NIAAA

Others: D. Goldman Chief LNG, NIAAA

M. Urbanek IRTA Fellow LNG, NIAAA

A. Novoradovsky Visiting Associate LNG, NIAAA

COOPERATING UNITS (if any)

University of New Mexico (F. Romero); Indian Health Service (C. North)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Human Neurogenetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

This project was not active during FY 94. It will be resumed for FY 95.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00018-02 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Statistical Genetics of Linked Multi-Allelic Loci

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Long Special Expert LNG, NIAAA

COOPERATING UNITS (if any)

Arizona State University (R. Williams)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Human Neurogenetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Haplotype and linkage disequilibrium analysis has contributed to studies on recombination and human gene mapping. This project develops an algorithm to obtain maximum likelihood estimates of haplotype frequencies from phenotypic data, and it develops a set of hypotheses that can be statistically tested in order to demonstrate pairwise and higher order linkage disequilibrium. A computer program was written to implement the methods. The utility of the methods and computer program is verified using Monte Carlo simulations. This work will be applicable to the many short tandem repeat DNA polymorphisms that our lab is typing for linkage analyses.

Project Description:Investigators:

J. Long	Special Expert	LNG, NIAAA
R. Williams	Professor	Arizona State University

Objectives:

Haplotype frequencies and linkage disequilibrium coefficients are difficult to estimate from population data because linkage phase cannot be deduced from multiple locus phenotypes. Moreover, highly polymorphic systems (e.g., short tandem repeat loci) can have many more possible haplotypes than would be contained in a large sample; a population may not even possess all possible haplotypes. Nonetheless, multiple locus phenotype frequencies contain information on haplotype frequencies, and the goal of this project is to utilize this information. The specific objectives are to: (1) develop a reliable algorithm for maximum likelihood estimation of haplotype frequencies for linked multi-allelic loci, (2) provide an easily implemented computer program to perform the estimation, (3) develop a hypothesis testing strategy to test the significance of observed linkage disequilibrium, and (4) implement computer simulations to determine the statistical distributions of the proposed test statistics.

Methods Employed:Estimation

Maximum likelihood haplotype frequency estimates are obtained from multiple locus phenotypes using an iterative Estimation-Maximization (E-M) algorithm. The expected number for every kind of haplotype is counted for each individual sampled, and these expectations are averaged over the entire sample. Our method is simplified relative to other E-M methods by evaluating for each individual only those haplotypes which could have contributed to their phenotype. Maximum likelihood estimates of allele frequencies and linkage disequilibria are obtained by algebra from the estimated haplotype frequencies.

Statistical Tests

With our method, linkage disequilibrium is tested for significance by comparing the likelihood of a general model including disequilibrium to the likelihood of a reduced model that assumes multiple locus equilibrium. The hypotheses recommended for testing are, H0: multiple locus equilibrium; H1: pairwise linkage disequilibrium without higher order disequilibrium (three-way); and H2: higher order disequilibrium. The test statistic is always twice the negative log likelihood ratio. An advantage to the likelihood ratio approach is that it allows the global equilibrium hypothesis (H0) to be partitioned into additive components for testing H1 and H2.

Computer Simulations

Monte Carlo methods were used to find the true distributions of test statistics under the various null hypotheses. Three locus phenotypes were simulated with allele frequencies and linkage disequilibrium patterned after a histocompatibility data set. Upon comparison to actual chi-square distributions, it was possible to identify the conditions which cause likelihood ratio statistics to deviate from their theoretical expectations.

Major Findings:

The methods described above were applied to three unlinked dinucleotide repeat loci in Navajo Indians, and to three linked HLA loci in Gila River (Pima) Indians. The likelihood functions of both data sets were shown to be maximized by the E-M estimates, and the testing strategy provides a useful description of

the structure of gametic disequilibrium. As expected, significant linkage disequilibrium was observed at all levels for the HLA data, but the dinucleotide repeat loci were apparently in equilibrium at all levels. Following these applications, a number of simulation experiments were performed to test how well the likelihood ratio statistic distributions are approximated by chi-square distributions. In most circumstances the chi-squares grossly underestimated the probability of Type I errors. This underestimation appears to be a function of unevenness of allele frequencies. However, the chi-square statistics also overestimated the Type I error probability at times. Overestimation occurred with smaller sample size.

Significance to Biomedical Research and the Program of the Institute:

Haplotype and linkage disequilibrium analysis has contributed to studies on recombination and gene mapping. Most recently, these techniques were employed in the identification of the Huntington's disease gene. A sound basis for statistical estimation and hypothesis testing is fundamental to any application of haplotype or disequilibrium analysis. Heretofore, methods and computer programs to estimate haplotype frequencies and linkage disequilibrium coefficients were not generally available. We can now reliably use the methods and program described here, and we are prepared to distribute the program for use in other laboratories.

Proposed Course:

We have submitted an abstract describing this research for presentation this fall at the American Society of Human Genetics meetings, and we have submitted a paper from this research to *The American Journal of Human Genetics*. Upon acceptance, the goals of this study will be accomplished and the study will be terminated.

Publications:

None.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00019-02 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

ALDH2 Deficiency: Population Genetics and Relationship to Phenotype

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Novoradovsky Visiting Associate LNG, NIAAA

Others: D. Goldman Chief LNG, NIAAA

COOPERATING UNITS (if any)

Laboratory of Parasitic Diseases, National Institute on Allergy and Infectious Diseases, NIH (T. Nutman, P. Zimmerman)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Human Neurogenetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In Orientals, ALDH2 deficiency due to a common polymorphism frequently causes a flushing reaction after alcohol consumption and this aversive reaction is responsible for lower rates of alcoholism in individuals with the inactive ALDH2-2 allele. ALDH2 deficiency was detected in South American Indian populations; however, these findings were never confirmed and a search for the Oriental ALDH2-2 allele in South American Indians was negative. In this project, we have: (1) confirmed absence of ALDH2 activity in 3,139 Chachi South American Indians, and (2) shown that the Oriental ALDH2-2 allele is either not present or present in low abundance across five populations of South American Indians.

This project was previously titled, "Molecular Genetic Study of Mitochondrial Aldehyde Dehydrogenase".

Project Description:Investigators:

A. Novoradovsky	Visiting Associate	LNG, NIAAA
D. Goldman	Chief	LNG, NIAAA
J. Kidd		Yale University
K. Kidd		Yale University
T. Nutman	Chief	LP, NIAID
P. Zimmerman		LP, NIAID

Objectives:

Mitochondrial aldehyde dehydrogenase (ALDH2) catalyzes the oxidation of short chain aldehydes to carboxylic acids, with the reduction of NAD. ALDH2 plays a key role in ethanol metabolism by irreversibly oxidizing acetaldehyde. This process is engaged mainly in liver, where ALDH2 action is highest. However, ALDH2 is also expressed in other tissues including hair follicles. Although the physiological role of ALDH2 in hair root cells is unknown, the expression of ALDH2 in hair roots provides a convenient means for population screening for ALDH2 deficiency which does not necessitate sampling of liver tissue.

Flushing due to aldehyde intoxication commonly occurs in Oriental populations after alcohol challenge. In these populations, flushing is due to a hereditary dominant deficiency of ALDH2, caused by an ALDH2<sup>2</sup>-allele, characterized by G>A transition in the exon 12 of the gene.

So far no other functional polymorphisms of ALDH2 have been identified, but other populations may have different variants. Among three South American Indian populations - Atacamenos (Chile), Mapuche (Chile), and Shuara (Ecuador) - approximately 40% were ALDH2-deficient (Goedde et al., 1986). We tested for presence of the ALDH2<sup>2</sup> allele using an allele-specific amplification assay (ASA), in a sample of 35 individuals belonging to seven South American Indian tribes. Many of these individuals were evaluated by RT-PCR and SSCP for ALDH2 sequence variants. To confirm that some South American Indians have ALDH2 deficiency, we evaluated hair root samples from Chachi Indians (Ecuador).

Methods Employed:

Hair root sampling was performed on 50 unrelated individuals of both sexes (age range 2-50 years, mean=22.7±2.4). All subjects belong to the Chachi Indian tribe, which is located in the Esmeraldo Province of Ecuador. Samples were collected by Drs. T. Nutman and P. Zimmerman.

Lymphoblastoid cell lines from 35 individuals from seven North American, Central American, and South American Indian populations were provided by Drs. J. Kidd and K. Kidd.

Biochemical genetic methods included isoelectric focusing (IEF), specific staining for aldehyde dehydrogenase (ALDH2), and two control enzymes: malate dehydrogenase (MDH) and phosphoglucomutase (PGM). The latter two enzymes served as controls for the stability of mitochondrial and cytoplasmic enzymes in extracts. For direct gene analysis to detect novel ALDH2 DNA substitutions, we used RT-PCR-SSCP of mRNA from lymphoblastoid cell lines. Direct sequencing of cDNA was also performed on some individuals.

Major Findings:

Reliability of ALDH2 phenotyping using hair roots presents technical difficulties because it depends on variations in hair morphology and condition. Therefore, the control enzymes MDH and PGM were assayed to ensure the accuracy of phenotyping.

Visual analysis of stained gels revealed 15 out of 50 subjects who had either absence or relatively low levels of ALDH2. However, 11 of these samples also had low staining intensity of the control enzymes PGM and MDH. These 11 samples reflect inadequate sampling conditions or insufficient number of anagen hairs. Therefore, we excluded those 11 samples from the sample size for assessment of population frequency of ALDH2 deficiency. Of the remaining 39 samples, three are characterized by decreased ALDH2 activity. We estimate frequency of ALDH2 deficiency in the Chachi Indians as  $7.7 \pm 4.3\%$ .

The identification of three Chachi Indians with absent ALDH2 activity is in accordance with previously reported data (Goedde et al., 1986) that South American Indians are characterized by a polymorphism of mitochondrial ALDH. However, the frequency of ALDH2 deficiency determined in Chachi Indians in our study is significantly lower than 42% which was reported by Goedde (1986) using a similar IEF technique among the Shuara Indians of Ecuador.

If this is due to interpopulation variation, then we could expect the wide range of allele frequencies between different tribes. Consequently, the apparent monomorphism of coding region of ALDH2 gene we found in 33 Indians, including five South American Indian tribes, could be explained by the absence of ALDH2-negative phenotypes among the individuals tested. However, this direct analysis for mutations using RT-PCR-SSCP is not 100% efficient and does not assay for functional variation. Assuming that the proposed allele of ALDH2 deficiency in South American Indians is inherited dominantly, as the ALDH2<sup>1</sup> gene, and allele frequency 0.04-0.23, based on the 0.07-0.4 incidence of enzyme deficiency in the South American Indians, the probability of 33 tested samples being normal homozygotes is  $(1-q)^{2 \times 33}$ , i.e., falls between  $3.2 \times 10^{-8}$  and  $6.7 \times 10^{-2}$ . In fact, it is probably even higher due to complex character of the studied sample, composed by small subsamples of representatives from different populations. If all samples tested so far by molecular genetic methods happen to be normal homozygotes, then there is a chance to find the proposed polymorphism after a preceding selection of ALDH2-deficient Indians. Thus, the results of IEF assay in hair roots, which allowed to select three ALDH-2 negative Chachi Indians from Ecuador, increase the probability that we may discover a novel deficient ALDH2 in South American Indian populations.

#### Significance to Biomedical Research and the Program of the Institute:

These studies demonstrate both that South American Indians have ALDH2 deficiency, as reported and that this deficiency has different molecular genetic origins from the "Oriental" allele ALDH2<sup>2</sup>. It also is an important step in the search for the "novel" silent allele in ALDH2 locus because we have now concentrated our efforts on individuals who are indeed ALDH2-negative.

#### Proposed Course:

We will perform direct analysis of the coding region of the ALDH2 gene in proven ALDH2-negative individuals in order to clarify the molecular origin of ALDH deficiency in South American Indians. This study will be carried out using the most rapid and powerful mutation screening strategies, including PCR-SSCP of DNA and/or cDNA fragments with ongoing sequencing.

#### Publications:

None.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00085-01 LNG

PERIOD COVERED

October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Search for the DNA-Expansion Mutations Among Alcoholic Patients

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Novoradovsky Visiting Associate LNG, NIAAA

Others: D. Goldman Chief LNG, NIAAA

COOPERATING UNITS (if any)

Clinical Neurogenetics Branch, NIMH (P. Gejman, Q. Cao)

LAB/BRANCH

Laboratory of Neurogenetics

SECTION

Section of Human Neurogenetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Bethesda, MD 20892-8205

TOTAL STAFF YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Expanded simple sequence (thus far, trinucleotide) repeats cause seven known diseases, all of which are neurogenetic. Expansion of repeats provides a mechanism whereby deleterious alleles may be rapidly generated in the general population. Therefore, it is important to determine if such alleles are associated with subtypes of alcoholism. We are seeking to improve the sensitivity of detection of expanded simple sequence repeats and to make the process more efficient. In this recently initiated project, we have developed a set of reliable positive and negative standards for repeat expansion detection (RED) using thermostable DNA ligase. Thus far, using only standards, we have successfully adapted RED to nonradioactive detection using fluorescently tagged oligonucleotide.



Project Description:Investigators:

A. Novoradovsky	Visiting Associate	LNG, NIAAA
P. Gejman	Visiting Scientist	CNB, NIMH
D. Goldman	Chief	LNG, NIAAA
Q. Cao	Visiting Fellow	CNB, NIMH

Objectives:

DNA instability, caused by amplification of trinucleotide repeats in human genome, has been proven to cause a number of neuro- and muscular degenerative disorders in man. To date there are seven known diseases of this nature: myotonic dystrophy, fragile X syndromes, Huntington disease, spino-bulbar muscular atrophy, spinocerebellar ataxia type I, and dentato-rubral-pallidoluysian atrophy. Their common features are the impairment of neural tissue, especially in the most severe cases, and a phenomenon of genetic anticipation, i.e., increased disease severity and earlier onset in successive generations. The anticipation is associated with increased length and meiotic and mitotic instability of repetitive trinucleotide stretches in the respective genes.

It is difficult to demonstrate anticipation in alcoholism in man, since its penetrance depends not only on predisposing genetic background, but also on certain predisposing environment. One of possible approaches to genetic research on alcoholism employs a genetic study of mental disorders, which demonstrates a comorbidity with alcohol and drug abuse, such as affective disorders, including depression and panic disorder, and antisocial personality disorder. Recent discoveries of anticipation in bipolar affective disorder (McInnis et al., 1994) and schizophrenia (Bassett et al., 1994) raise an issue of search for potential trinucleotide expansions as a possible genetic basis of the most severe cases of alcoholism and related illnesses. High occurrence of polymorphic trinucleotide repeats in human genome provides a necessary background for possible triplet expansions, and can additionally justify for this dynamic mutations search in the study of alcoholism.

The existing methods for the novel DNA-expansion mutations - Southern blotting and oligonucleotide ligation assay, or repeat expansion detection (RED) - suffer from insufficient reliability, high time and labor expenditure, and bears a potential environmental risk because of high amounts of radioactive labels needed. In order to perform a large-scale screening of clinical populations, we have to improve the detection technique. Our objectives are: eliminating the blotting step, switching to nonradioactive detection, and creation of reliable positive and negative reaction standards.

Methods Employed:

The methods include synthesis of fluorescently-labeled oligonucleotides with intact 5'- and 3'- termines, necessary for the ligation assay; repeat expansion detection (RED) using thermostable ligase; and electrophoresis in polyacrylamide gels using the Model 373 DNA Sequencer (ABI) for detection of fluorescent ligation products.

Major Findings:

A concatemer chain reaction (CCR) method was developed to control templates for the ligation of oligonucleotides. The method capitalizes on the ability of tandemly repetitive complementary pairs of oligonucleotides, such as (CTG)<sub>n</sub> and (CAG)<sub>n</sub>, to partially anneal with formation of single-stranded overhangs. These overhangs are filled by DNA polymerase during thermal cycling. PCR of such pairs of oligonucleotides results in a series of tandem triplet duplexes with mean

length depending on number of cycles. The 1-9 Kbp products of CCR were isolated (length was assessed by 0.8% TAE agarose electrophoresis) and mixed with human genomic DNA from healthy individuals to a final concentration equivalent to a single gene copy. These mixtures served as positive controls for the ligation reactions.

Two approaches were developed for synthesis of fluorescently-labeled oligonucleotides. First, fluorescent (CTG)<sub>17</sub> oligo was synthesized with incorporation of Fluorescein-ON phosphoramidite (CLONTECH) into one or several internal positions. Second, we internally incorporated a Uni-link Amino Modifier (CLONTECH) - modified phosphoramidite coupled with an eight-carbon chain, ended by a primary amine group. Subsequently, fluorescent dye was attached to this functional group. Use of oligonucleotides prepared by the first approach gave positive results with high template concentrations, but this approach gave a low yield of full-length oligonucleotides. Currently, we are testing the second approach to the synthesis of fluorescent oligonucleotides.

#### Significance to Biomedical Research and the Program of the Institute:

To date there are no data on the possible contributions of relatively "new" forms of human mutation - dynamic mutations - to individual predisposition to complex behavioral phenotypes such as alcoholism. The planned large-scale search of unstable expanded microsatellite DNA in alcoholic and other psychiatric and neurological patients will help both to elucidate this question and to further explore the phenomenon of DNA instability in human disease and behavior.

#### Proposed Course:

We will continue to develop methods for preparing fluorescently-labeled oligonucleotides for the ligation assay. We will create a series of ten oligonucleotide pairs, corresponding to the ten possible nucleotide combinations that might occur in expanded repeats, and we will generate the appropriate artificial control templates. Mitotic instability of tandem dinucleotide domains in human genome has also been demonstrated in certain tumors and in cultured human lymphoblasts. Therefore, we also will make analogous preparations for a dinucleotide repeat expansion search.

In the main stage of the project, we will carry out a screening for the dynamic mutations, involving triplet expansion, in a series of DNA samples of alcoholics, and from patients with related disorders. In order to clarify which of ten possible triplet combinations are more inclined to mitotic instability, we will compare different trinucleotide microsatellites in the DNA samples from lymphocytes and respective lymphoblastoid cell lines after several passages.

#### Publications:

None.



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